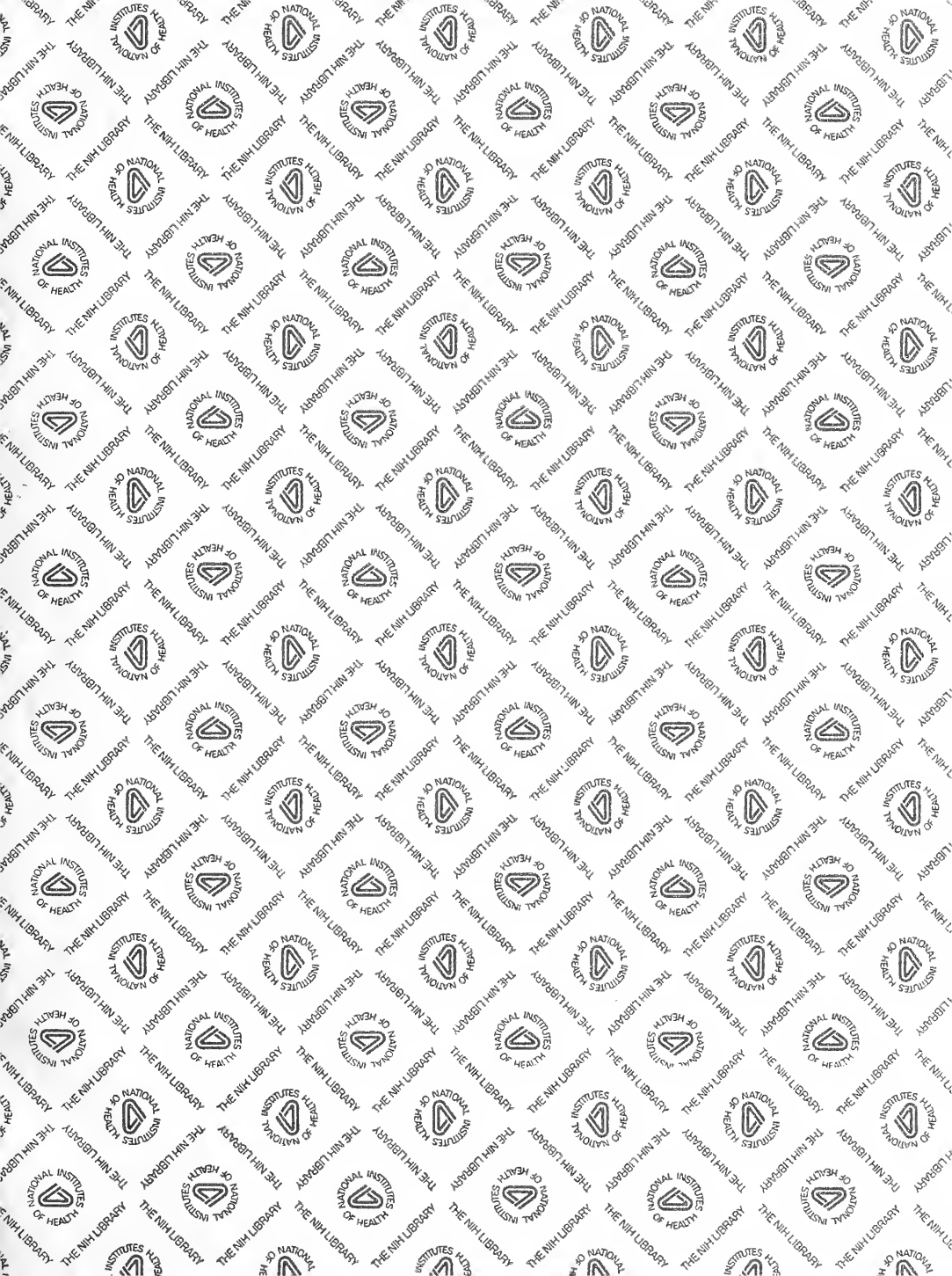
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ANNUAL REPORT OF THE SCIENTIFIC DIRECTOR, NHLBI
OCTOBER 1, 1993 - SEPTEMBER 30, 1994

The Division of Intramural Research, NHLBI, consists of 17 Laboratories and Branches working on a wide range of projects. The following is a brief summary of some of the highlights of their work this last year.

The Cardiology Branch has obtained additional support for its hypothesis that formation of the neointimal lesion in blood vessels during restenosis results from alterations that confer upon cells a selective growth advantage that leads to excessive proliferation. It studied atherectomy tissue from the excised restenotic lesions of patients and demonstrated that 38% of patients exhibited elevated levels of the tumor-suppressor protein, p53, in the smooth muscle cells of the lesions. Of lesions with elevated p53, 85% had human cytomegalic virus sequences, identified by PCR. Smooth muscle cells grown from the lesions expressed the HCMV viral protein, IE2, and exhibited elevated p53 levels. HCMV infection of normal vascular smooth muscle cells in culture increased p53 accumulation, which correlated temporally with IE2 expression. Thus, HCMV and IE2 mediate inhibition of p53 function, which may contribute to the development of restenosis.
The Cardiology Branch, in order to see if gene therapy might prevent restenosis, has developed an adenovirus vector encoding the herpes simplex gene thymidine kinase (HS-tk), which when expressed in cells, renders them sensitive to the drug, ganciclovir. It demonstrated that transfection with HS-tk, followed by ganciclovir treatment, inhibits smooth muscle proliferation in vitro and in a rat model of balloon injury. It is proceeding to larger, more complex animal models that more closely simulate the human lesion.

The Hematology Branch previously identified the human erythroid progenitor cell as the target cell of B19 parvovirus. Now it has described a new parvovirus syndrome due to congenital infection. When fetuses are infected in the mid-trimester, hydrops fetalis results. When interventional treatment with blood cell transfusions is administered either in utero or at birth, infants may be born with severe anemia, characterized morphologically as either pure red cell aplasia (Diamond-Blackfan syndrome) or a congenital dyserythropoietic state. The researchers have shown that these infants are infected with B19 parvovirus, but low levels of virus are restricted to the bone marrow. Treatment with immunoglobulin, ordinarily effective in persistent infection, is not helpful. A secondary immune mechanism may suppress hematopoiesis in these infants.

In the new Bone Marrow Transplant Unit, which opened in 1993, the Hematology Branch has now performed a total of nine allogeneic transplants for chronic myelogenous leukemia and myelodysplastic syndromes. Five of six patients transplanted from fully matched donors have survived without leukemia.
Three patients have died, two following failed engraftment from partially matched donors, and one from graft vs. host disease and infection. The Hematology Branch has completed its study of intensive immunosuppression with a combination of

antithymocyte globulin (ATG) and cyclosporin to treat 55 patients with severe aplastic anemia. It has obtained a hematologic response rate of 71% at three months without treatment-related mortality. Both the response rate and survival rate are far superior to the historical control population which was treated with ATG alone. Response rates with intensive immunosuppression were excellent both for children and severely neutropenic patients, two populations previously resistant to conventional immunosuppressive therapy.

The Hypertension-Endocrine Branch has developed, in collaboration with Dr. David Goldstein, NINDS, a new test for the diagnosis of pheochromocytoma. They used high-pressure liquid chromatography and electrochemical detection to measure metanephrines in blood. They found that a pheochromocytoma resulted in considerably larger increases in plasma metanephrines than in catecholamines. The test showed 100% sensitivity, and greater specificity than plasma catecholamines. The finding of normal plasma metanephrines virtually excluded the diagnosis of pheochromocytoma. Therefore, measurement of plasma metanephrines provides the most sensitive single clinical test yet described for the diagnosis of pheochromocytoma.

The Pathology Branch has completed studies on the dilated cardiomyopathy associated with chronic Chagas² disease that demonstrated the presence of large quantities of laminin, which is present not only in the thickened basement membranes, but also in amorphous deposits in the myocardial interstitium. This is of importance, since other studies have shown that sera from patients with chronic chagasic cardiomyopathy have high titers of circulating antibodies against laminin. This suggests that the disease may be an autoimmune reaction, since laminin-like molecules have been demonstrated on the surfaces of the parasite responsible for Chagas² disease. The Pathology Branch has also shown, using beagle dogs, that the administration of ICRF-187 can essentially ameliorate the cardiac toxicity of doxorubicin. The cardiotoxicity induced by the doxorubicin family of chemotherapeutic agents results from the formation of doxorubicin-iron complexes that catalyze the generation of oxygen-free radicals. ICRF-187 appears to function as an iron chelator, thereby decreasing the formation of free radicals and the resultant cardiotoxicity.

The Pulmonary Branch performed the first human studies of gene therapy for cystic fibrosis. It administered an adenovirus vector which had been modified with the normal cystic fibrosis transmembrane regulator (CFTR) gene to the nasal and bronchial epithelium of five patients with cystic fibrosis. It was able to demonstrate the successful transfer of the CFTR cDNA and the presence of the CFTR protein to the nasal or bronchial epithelium of some of the patients. There was no evidence of recombination or complementation of the vector to produce a replication-competent adenovirus, or shedding of the vector, or rise in titers of serum anti-adenovirus neutralizing antibodies. In the fifth patient, at the highest dose, there was a transient systemic and pulmonary syndrome, including headache, fatigue, fever, tachycardia, dyspnea, hypoxemia, pulmonary infiltrates,

and hypotension, likely secondary to local inflammation initiated by the vector in the lower respiratory tract. Findings suggested that this reaction was mediated by interleukin-6. Dr. Ronald Crystal has moved to Cornell University, New York City, where he will continue these studies.

The Pulmonary Branch has developed an animal model to evaluate the role of nitric oxide (NO) in the pathogenesis of various lung diseases. It studied NO production by the lung in male Sprague-Dawley rats given lipopolysaccharide from E. coli. It was able to demonstrate induction of nitric oxide synthase in animals injected with endotoxin, but not in controls. It also demonstrated the metabolic products of NO as nitrates in both the supernatant from alveolar macrophages in vitro, and in lavage fluid from animals treated with the endotoxin.

The Laboratory of Biochemistry has used the enzyme, glutamine synthetase, from E. coli to show that nitration of proteins, whose activities are under strict control by phosphorylation-dephosphorylation of tyrosine residues, can seriously compromise their regulatory properties. The irreversible nitration of specific tyrosine residues was due to peroxynitrite produced in vivo by reaction of nitric oxide with superoxide anions. This is a specific demonstration of oxidative injury in an important biological system. It has also shown that reversible oxidation of specific cysteine residues on the protease of human immunodeficiency virus (HIV) completely inactivated it, indicating that oxidation may be important in the regulation of HIV protease activity and replication.

The Laboratory of Biochemical Genetics has developed a mammalian, spinal cord neuron-skeletal muscle culture system in which neurites induce postsynaptic-like accumulations of acetylcholine receptors on the myotubes, after as little as six hours of co-culture. It has shown that this inductive ability is predominantly a property of developing axons, as opposed to dendrites, and that the axon-myotube contacts can develop accumulations of synaptic vesicles, a typical synaptic cleft, and a differentiated post-synaptic cell surface. This new system will allow studies of the role of various putative signals in postsynaptic receptor aggregation and of other mechanisms of synaptogenesis.

The Laboratory of Biochemical Genetics has also developed a model with which to study the activation of apoptosis or internally programmed cell death for neurons. It uses low-dose ionizing irradiation of pregnant rats of 16-18 days gestation, which causes rapid and massive death of proliferating and differentiating stem cells of the fetal forebrain. It was found that gamma radiation elicits, within three hours, nuclear pyknosis and fragmentation in undifferentiated cells in the cortical neuroepithelium, as well as fragmentation of fetal brain DNA into an oligo-nucleosomal ladder pattern characteristic of apoptosis. This DNA fragmentation requires ongoing RNA and protein synthesis and indicates that the radiation kills these cells via the mechanism of apoptosis.

The Laboratory of Biophysical Chemistry, in collaboration with the Laboratory of Biochemical Genetics, has completed the three-dimensional structure of the 77 amino acid homeodomain protein NK-2 from Drosophila. It has also used its recently discovered pH zone refining countercurrent chromatography to successfully separate gram quantities of enantiomers of a leucine derivative, using a chiral selector in the stationery phase. This technique is of great interest to the pharmaceutical industry that seeks to prepare pure enantiomers of chiral drugs. Chiral forms of thalidomide tend to assume achiral structures. And, despite its deleterious effects on fetal development, thalidomide appears to have promise in AIDS therapy.

The Laboratory of Cardiac Energetics has shown that ATP, ADP, and inorganic phosphate (Pi) levels are highly buffered by oxidative phosphorylation in the healthy myocardium and that these metabolites are unlikely to play an important role in the orchestration of metabolism or coronary blood flow. Its work has shown that NADH is only reporting the force driving oxidative phosphorylation, and is not significantly involved in the kinetic control of the latter. These data are consistent with the linear relation between NADH and respiratory rate and the lack of cooperativity between NADH and ADP. It has also shown that adenosine does not play a significant or necessary role in the regulation of coronary blood flow with increases in work. Using the 4 Tesla NMR magnet system, the Laboratory has collaborated with the Cardiology Branch to show that specific alterations in cardiac myosin in patients with hypertrophic cardiomyopathy are reflected in metabolic deficiencies in the soleus muscle. This has led to new hypotheses as to why a genetic modification of myosin results in an apparent metabolic compromise of skeletal muscle.

The Laboratory of Cell Biology has continued its studies of non-muscle myosin and shown that in Acanthamoeba myosin I heavy chain kinase can be fully activated by binding to phospholipid vesicles containing 15-30% phosphatidylserine before there is significant autophosphorylation. Activation of kinase by either phospholipids or autophosphorylation involves an increase in Vmax with no change in Km for myosin I. A specific role for certain phosphomyosins in contractile vacuole function was shown. The laboratory has also shown that the uncoating of clathrin baskets by the bovine brain heat shock protein (hsp70) requires the presence of a 100-kDa protein cofactor which may be the previously described assembly protein, auxilin.

The Laboratory of Cellular Metabolism has demonstrated the similarity between the appropriate counterparts of each of the three mammalian ADP-ribosylation factors (ARF) classes in Drosophila. It believes that this experimental organism will aid in the elucidation of the role of ARF²s in intracellular vesicular transport.
The Laboratory has also identified NAD:arginine ADP-ribosyltransferase mRNA and protein in both skeletal and cardiac muscle, but not in other tissues, from several mammalian species. Collective expression of ADP-ribosyl transferase in integrin A7 in cardiac and skeletal muscle, their parallel developmental appearance, and the

apparent specificity of ADP-ribosylation are consistent with a regulatory association between these two proteins. Integrin A7, in association with integrin B1, is a laminin-binding protein. Laminin is the substance found by the Cardiac Pathology Branch to be highly associated with the cardiotoxicity in Chagas[2] disease.

The Laboratory of Chemical Pharmacology has shown that stimulation of rat mast cells via the adenosine A3 receptor elicits transient activation of phospholipase C and mitogen-activated protein (MAP) kinases, but sustained activation of phospholipase D and protein kinase C. The secretory response to antigen can be reconstituted in washed-permeabilized cells by provision of physiologic concentrations of calcium and certain isozymes of protein kinase C. This indicates that these two elements transduced the required signals for secretion. Phospholipase D provides a reinforcing signal, probably through the generation of diacylglycerols which activate protein kinase C.

The Molecular Disease Branch has shown that both lipoprotein lipase (LPL) and hepatic lipase (HL), two enzymes that are vitally important in the regulation of fat metabolism, contain loops of amino acids or "lids" that play a pivotal role in determining their substrate specificity. It showed that hypoalphalipoproteinemia is a heterogenous condition, that not all patients with low HDL have an increased risk of premature cardiovascular disease, and that the metabolism of LpA-I and LpA-I:A-II are affected independently in patients with low HDL levels. This provides a new approach to the identification of which individuals with low HDL are at increased risk of premature cardiovascular disease. It showed that the LDL receptor does not play a major role in the catabolism of Lp(a), and therefore, the increased plasma levels of Lp(a) in FH must be due to increased production. The Branch has discovered a new molecular defect responsible for cholesterol ester transfer protein (CETP) deficiency in a patient and this provides new information about the heterogeneity of molecular defects leading to CETP deficiency. It has prepared a transgenic mouse model to evaluate the role of lecithin cholesterol acyltransferase (LCAT) in HDL metabolism. These studies have indicated that LCAT overexpression provides protection against diet-induced atherosclerosis in this mouse model.

The Molecular Hematology Branch has succeeded in transferring plasminogen activator genes into primate endothelial cells, and then seeded these cells onto thrombogenic vascular graft segments and shown increased tissue plasminogen activator (t-PA) and urokinase secretion by these cells. In an in vivo baboon thrombosis model, it found that platelet and fibrin deposition were significantly decreased by seeding of graft segments with genetically modified endothelial cells expressing either t-PA or urokinase. It could demonstrate in vivo retention of genetically modified endothelial cells for at least two hours following placement of the seeded grafts in the arterial circulation of sheep.

The Laboratory of Kidney and Electrolyte Metabolism has shown that collecting duct water permeability is regulated by vasopressin, via both short-term and long-term mechanisms. It used infusions of vasopressin into Brattleboro rats, i.e., rats with an absence of circulating vasopressin. There was nearly a three-fold increase in water channel expression that correlated with a nearly three-fold increase in vasopressin-stimulated water permeability in the isolated perfused collecting ducts from the rats. Direct verification was provided of the "shuttle hypothesis", i.e. regulation of plasma membrane water permeability occurs via regulated exocytosis of vesicles containing water channels. Additional data indicated that ATP decreases water permeability through a protein kinase C-mediated decrease in cyclic AMP production. Water permeability was decreased by the protein kinase C inhibitor, calphostin C.

The Laboratory of Kidney and Electrolyte Metabolism has identified large and variable amounts of organic osmolytes in rat and rabbit renal intermedullary cells, namely sorbitol, inositol, glycerophosphorylcholine, taurine, and betaine. Accumulation of such organic osmolytes in response to osmotic shock is a basic biological phenomenon, previously identified only in cells ranging from bacteria to cells of lower vertebrates. The present recognition of the vital role of organic osmolytes in the renal medulla is the first indication that it is more than a curiosity in mammalian cells.

The Laboratory of Pulmonary and Molecular Immunology has discovered that the gamma chain of the IL-2 receptor is common to multiple cytokine receptors, i.e., IL-4, IL-7, and IL-9 receptors. This recognition provides a much clearer understanding of why the defect in x-linked severe combined immunodeficiency (XSCID) in humans is so severe. The Laboratory originally predicted that less severe phenotypes might result from other γc mutations. Now it has found that γc is also the genetic defect in a moderate x-linked combined immunodeficiency (XCID). These studies have provided a clearer direction to achieving prenatal and postnatal diagnosis, carrier female identification, and gene therapy for XSCID.

A number of important appointments and organizational changes occurred this year. Dr. Joel Moss, previously Deputy Chief of the Laboratory of Cellular Metabolism, was appointed Chief of the re-named Pulmonary-Critical Care Medicine Branch. The Laboratory of Cellular Metabolism was abolished and its three Sections incorporated into the Pulmonary-Critical Care Medicine Branch with Dr. Martha Vaughan, previously Laboratory Chief, becoming Deputy Chief of the Branch.

Dr. James Gillette retired as Chief of the Laboratory of Chemical Pharmacology and was replaced by Dr. Warren Leonard, previously Chief of the Pulmonary and Molecular Immunology Section. The new Laboratory, which has been re-named the Laboratory of Molecular Immunology, includes all of the personnel of the old Laboratory. Dr. Earl Stadtman has resigned his position as Chief, Laboratory of

Biochemistry to devote full time to research. Dr. Boon Chock has been appointed Laboratory Chief. The Section headed by Dr. Sue Goo Rhee has been made into an independent Laboratory of Cell Signalling with Dr. Rhee as Laboratory Chief. Finally, the Pathology Branch has been re-organized as a Pathology Section under Dr. Victor Ferrans in the Office of the Director of Intramural Research.

ANNUAL REPORT OF THE CARDIOLOGY BRANCH
National Heart, Lung, and Blood Institute
October 1, 1993 through September 30, 1994

The experimental interests of the Cardiology Branch have continued to evolve over the past year as a result of an expanded section on molecular biology, adding the Section on Inherited Cardiac Diseases, and maintaining our longstanding efforts in clinical and basic physiologic investigations.

VASCULAR BIOLOGY AND CORONARY RESTENOSIS

Increasing evidence implicates the smooth muscle cell (SMC) in the process of atherogenesis. Thus, it appears that SMCs are activated in response to injury, causing their proliferation and migration from the media to the intima. A similar but markedly accelerated process causes restenosis following coronary angioplasty in 25-50% of pts wks or months later. The focus of the research efforts of the Section on Molecular and Cell Biology is twofold: 1) to derive insight into the basic mechanisms responsible for the restenosis process and, 2) to develop novel molecular-based strategies to prevent restenosis after angioplasty.

Viruses and viral-mediated mechanisms as contributors to the development of restenosis: On the basis of an earlier proposal that atherosclerosis (also characterized by SMC proliferation) might be a form of benign neoplasia, we hypothesized that formation of the neointimal lesion during restenosis may be driven by alterations that confer to cells a selective growth advantage; upon activation, as by injury, such cells would undergo excessive proliferation. We investigated two molecular mechanisms that might contribute to the abnormal SMC proliferation: *(i)* aberrant expression of p53, a tumor suppressor protein that inhibits cell cycle progression and that is functionally inactivated in many human cancers, and *(ii)* activation of latent HCMV, a herpesvirus that has been associated with the development of atherosclerosis. Conceivably, an interaction between an HCMV protein(s) and p53 could impair the latter's growth suppressor function, as is the case for proteins encoded by several DNA tumor viruses.

Our studies of the atherectomy tissue of the restenosis lesions demonstrated that 38% of pts exhibited elevated levels of the tumor suppressor protein p53 in the lesion's SMCs--sequencing revealed the p53 to be wild-type. Of lesions with elevated p53, 85% had human cytomegalovirus (HCMV) sequences in the lesions (by PCR). SMCs grown from lesions expressed the HCMV viral protein IE2 and exhibited elevated p53 levels. HCMV infection of normal cultured SMCs increased p53 accumulation, which correlated temporally with IE2 expression. We further demonstrated that IE2 directly binds with p53 and abolishes p53's ability to transactivate a reporter gene. Thus, HCMV and IE2 mediated inhibition of p53 function may contribute to the restenosis development. Because restenosis shares many features with atherogenesis, and atherosclerotic vessels often contain HCMV, HCMV-mediated inhibition of p53's growth suppressor function may also play a role in atherogenesis.

Gene therapy: Major efforts have focused on the use of adenoviral vectors to gain mechanistic insight and perhaps therapeutic benefit in vascular disease. The increased efficiency of adenoviral vectors over previous methodology, such as retroviruses or lipo-

somes, make them attractive for genetic manipulation of primary cells in culture as well as a useful vehicle for gene transfer in vivo. Using adenoviruses encoding the histochemical marker gene ß-galactosidase, over the last year we demonstrated that adenoviral vectors increase the efficiency of gene transfer into adult myocardium or the injured vessel wall by 1-3 orders of magnitude over previous methods. We subsequently used an adenovirus encoding the herpes simplex gene thymidine kinase (HS-tk), which when expressed in cells renders them sensitive to the drug ganciclovir. We demonstrated that HS-tk transfection followed by ganciclovir treatment inhibits SMC proliferation in vitro, and in a rat model of balloon injury. These studies are currently proceeding using larger, more complex animal models that more closely simulate human lesions.

Signal transduction pathways We have also used adenoviral vectors to better understand signal transduction in cells of the vessel wall. Over the last year we have examined whether reactive oxygen intermediators (ROI) such as superoxide and hydrogen peroxide play a role in cell signalling. Using adenoviruses that encode the scavenger enzymes superoxide dismutase and catalase, we have been able to alter ROI metabolism in SMCs and in endothelial cells. Preliminary results suggest that ROIs may modulate both growth factor stimulation and apoptosis in these cells.

ENHANCEMENT OF CORONARY COLLATERAL DEVELOPMENT

CAD pts who develop ischemic symptoms refractory to pharmacologic therapy and who are not candidates for bypass surgery pose a major therapeutic problem. Several years ago we postulated that coronary collateral development could be enhanced by angiogenic peptide growth factors and initiated animal studies using two peptides, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), to test this hypothesis.

The first studies of the Section of Experimental Physiology demonstrated that in dogs in which gradual coronary occlusion was produced, intracoronary bFGF or VEGF for 20 days increased collateral flow by approximately 40% after one month. In a second experiment, we found that it was not necessary to give bFGF directly into the coronary vessels; 20 days of systemic bFGF administration substantially accelerated collateral development. In dogs that received systemic bFGF for 5 or 9 weeks, we again found that bFGF enhanced collateral flow. Interestingly, there was no advantage to receiving bFGF for 9 vs. 5 weeks. In fact, the major improvement in bFGF-treated dogs was limited to the period 10-17 days after implantation of the constrictor, presumably the period of most intense ischemia. Subsequently we found that systemic administration of bFGF during this 10-17 day interval increased collateral flow by over 65%, with the benefit sustained three weeks after suspension of treatment.

This year, we have evaluated the long term (6 month) effect of bFGF on collateral blood flow, as well as the effect of the peptide on myocardial contraction. In addition, we are conducting studies to assess the minimum effective duration of bFGF treatment, and planning studies to ascertain the minimum effective dose. Studies to evaluate the potential use of gene transfer to deliver genes for angiogenic growth factors will be initiated soon. Finally, plans are being made for a Phase I clinical trial of bFGF in pts with CAD.

HYPERTROPHIC CARDIOMYOPATHY (HCM)

HCM is an inherited heart disease, characterized by cardiac hypertrophy, severe symptoms, and sudden death (SD). The studies of the Inherited Cardiac Diseases Section are directed at 1) improving risk stratification of such pts, 2) elucidation of mechanisms of SD and syncope, 3) relieving symptoms, 4) developing effective antiarrhythmic therapy, and 5) understanding the genetics of the disease and its pathophysiology at a molecular level.

About 25% of HCM pts have obstruction to LV outflow. We showed that DDD pacing improves symptoms and relieves the obstruction in pts with drug-refractory symptoms in most pts with obstructive HCM. Beneficial hemodynamic adaptive changes and regression of cardiac hypertrophy were noted following chronic pacing. This novel therapy is therefore an attractive alternative to cardiac surgery. A study is ongoing to determine whether this approach can prevent progression of the disease in children. We are also exploring a possible molecular basis for the regression of the cardiac hypertrophy.

With the report that the βMHC gene is responsible for HCM in some kindreds, attempts have been made to identify HCM kindreds with distinct βMHC gene mutations. We have identified 19 distinct mutations in the β-MHC gene located on chromosome 14, in 32 unrelated kindreds, and mutation-specific natural histories are being determined. Other families have been identified in which the disease is not linked to the β-MHC gene, and linkage studies are in progress to identify other genes that can cause HCM.

We have also performed genetic, histochemical, and functional analyses on skeletal muscle from pts with distinct βMHC gene mutations. We demonstrated that mutant messenger RNA and β-myosin are present in slow skeletal muscle of affected pts, and that skeletal muscle myofibers containing mutant β-myosin have abnormal contractile properties. We also found that β-myosin purified from skeletal muscle of pts with distinct βMHC gene mutations has abnormal function, as determined in an in-vitro motility assay. Histochemical analyses showed that 60% of pts with βMHC gene mutations have skeletal myopathy--specifically, "central core disease", a very rare, non-progressive myopathy characterized by loss of mitochondria from the center of some of the slow myofibers. Recently, the 3-dimensional structure of chicken skeletal muscle myosin has been described. Homology between this myosin and human cardiac myosin has allowed us to study the location of the mutations in terms of functional domains of the molecular motor.

CONTRIBUTION OF THE ENDOTHELIUM TO VASCULAR TONE, PLATELET FUNCTION, AND MICROVASCULAR DYSFUNCTION

The endothelium secretes substances that dilate vascular smooth muscle and inhibit platelet aggregation. The Section on Cardiac Catheterization and the Echo and Clinical Physiology Lab have continued their studies designed to elucidate 1) the regulation of microvascular function in the coronary and systemic circulation of humans, 2) the mechanisms by which the endothelium normally modulates coronary and systemic vascular tone and influences platelet function, and 3) how its dysfunction contributes to the development of various disease states.

Endothelial Function in Hypertension and Hypercholesterolemia: We have shown that pts with hypertension and pts with hypercholesterolemia have impaired endothelium-dependent vascular responses to acetylcholine (ACH), and that this abnormality is related to diminished activity of nitric oxide (NO), a substance produced by the endothelium that induces relaxation of the underlying smooth muscle and thus regulates vascular tone. NO is synthesized by endothelial cells from L-arginine under basal conditions and is rapidly broken down by superoxide anions. Our efforts have been directed to elucidating the basic mechanisms of abnormal NO activity in hypertensive and hypercholesterolemic pts. Thus, we showed that the abnormality is not related to decreased availability of L-arginine, the natural precursor of NO, and that it is not secondary to a specific defect at the level of the muscarinic receptor by demonstrating that the response to substance P (a non-muscarinic endothelial agonist) is also depressed in hypertensive and hypercholesterolemic pts. Utilizing bradykinin, an endothelial agonist that preferentially activates a different signal transduction pathway to stimulate the synthesis of nitric oxide, we found evidence of a selective abnormality at the level of the intracellular signal transduction pathways in pts with hypercholesterolemia. In contrast, in pts with essential hypertension, we found that the endothelial dysfunction is not limited to a specific signal transduction pathway; the defect, therefore, is either more generalized or involves a final common pathway in the nitric oxide signalling cascade.

Our current efforts are related to the investigation of an abnormality in the breakdown of nitric oxide by superoxide anions. In particular, we want to determine whether increased destruction of nitric oxide by superoxide anions contributes to the endothelial dysfunction we have demonstrated in hypertension and hypercholesterolemia.

Regulation of microvascular function in the coronary and systemic circulation of humans. In previous years we primarily investigated pts with angina-like chest pain and potential ischemic syndromes in the absence of epicardial CAD, demonstrating that a subset of this pt population has evidence for coronary microvascular dysfunction. Over the past 2 years we have focused our efforts on 2 groups of subjects: postmenopausal women and hypercholesterolemic subjects. We demonstrated that estrogen importantly affects endothelium-dependent vasomotion of both the coronary and the systemic (forearm) circulations of estrogen-deficient postmenopausal women when acutely administered. Estrogen also protects low density lipoprotein from oxidation when administered acutely and chronically to postmenopausal women. Over the past year we performed studies assessing whether the antioxidant effects of estrogen are responsible for its vasomotor effects.

Because oxidatively modified low density lipoprotein can adversely affect vascular responsiveness (at least in animal models with tissue examined ex vivo), we performed studies using antioxidant vitamins in hypercholesterolemic pts, a population with demonstrable vascular dysfunction. We also investigated the interaction of estrogen and vitamin E on oxidation of low density lipoprotein and forearm vascular responsiveness in postmenopausal women. Studies are also being conducted to investigate orally administered estrogens compared to transdermally administered estrogens with regard to low density lipoprotein oxidation and forearm vascular responsiveness. We are also conducting a study to look at the antioxidant and vascular effects of the antiestrogen tamoxifen. Preliminary data suggests that the vascular effects of estrogen administered at physiologic levels are mediated through potentiation of the production or release of nitric oxide. A study will begin shortly

to further clarify this mechanism of acutely administered estrogen. In the same study we will investigate whether estrogen's antioxidant effects are mediated through the release of nitric oxide. These studies will be performed both in the forearm and in the coronary circulations.

As we continue to perform studies of coronary microvascular dynamics using endothelium-dependent and independent agonists at the time of cardiac catheterization, we are attempting to demonstrate whether or not such studies identify pts with objective evidence of myocardial ischemia during stress using transesophageal echocardiography and dobutamine stress. We are also conducting studies to assess whether insulin resistance, a phenomenon commonly found in pts with chest pain and normal coronary angiograms, has any dynamic vascular consequences and whether pts with evidence for insulin resistance are more or less likely to have objective evidence of ischemia during stress. Because most pts with chest pain and normal coronary angiograms are women, we are also assessing the usefulness of transesophageal echocardiography with dobutamine stress to identify those pts with evidence of myocardial ischemia during stress, comparing this response to conventional noninvasive testing such as exercise electrocardiography, thallium scintigraphy, and radionuclide angiography. The responses of women to dobutamine stress will be compared to women with CAD using the same testing. We will attempt to show whether dobutamine stress testing has greater sensitivity and specificity than other conventional noninvasive testing in identifying women likely to have normal coronary angiograms at cardiac catheterization.

We are also interested in mechanisms of pain in pts with chest pain and normal coronary angiograms, based on our observation that as a group, these pts commonly have exaggerated or abnormal cardiac pain sensitivity. We are performing studies of pain pathway activation in the central nervous system using oxygen-15 labeled water and positron emission tomographic imaging both at rest and during dobutamine stress. In the future we intend to study the role of endogenous opioids in mediating activation of pathways in pts with chest pain and normal coronary angiograms compared to normal volunteers and pts with CAD with symptomatic angina and silent ischemia.

Relation between CAD risk factors and endothelial dysfunction: Over the past year we have demonstrated that pts with angiographically normal coronary arteries who are exposed to CAD risk factors (hypertension, hypercholesterolemia, etc.) exhibit endothelial dysfunction involving both the coronary epicardial vessels and microvessels that is not substantially different from the dysfunction observed in pts with established atherosclerosis. We also found that the level of endothelial dysfunction is related to the number of risk factors.

These findings have therapeutic implications. For example, studies in the cath lab have demonstrated in pts with CAD risk factors that endothelial dysfunction partially improves after administration of intracoronary L-arginine. To determine whether this translates into reduced myocardial ischemia, a study is in progress examining the effects on ischemia of L-arginine administration to pts with hypercholesterolemia and CAD. Additional studies are in progress and are planned to assess the pathophysiologic and therapeutic impact of impaired NO availability in human atherosclerosis. These include studies to determine: whether free radical scavengers reverse the endothelial dysfunction present in pts with risk factors for CAD; whether there is an interaction between the sympathetic nervous system and the NO system in the coronary and peripheral vasculature; the impact of endothelial dysfunction on NO-induced platelet inhibition; whether interventions designed to improve endothelial

dysfunction exert inhibitory effects on platelet aggregation; the impact of myocardial ischemia on endothelial function; the effects of new therapeutic strategies for improving endothelial function (L-arginine, antioxidant therapy) on development of ischemia, platelet dysfunction, LV dysfunction, atherosclerosis progression and, ultimately, on coronary event rate. In particular, over the past few years, coronary and femoral endothelial function has been studied in more than 150 pts. Follow-up of these pts over the next five-year period will provide invaluable information on the long-term consequences of endothelial dysfunction.

Coronary Artery Disease

In many pts with chronic CAD, impaired LV function at rest represent chronic hypoperfusion, termed hibernating myocardium. Identification of pts with such potentially reversible LV dysfunction has been problematic, as regional dysfunction arising from hibernating but viable myocardium may be clinically indistinguishable from that arising from infarcted myocardium. Since enhanced LV function after revascularization is associated with improved survival, prospective identification of viable myocardium in pts with CAD and LV dysfunction has important clinical and prognostic implications.

Identification of Viable Myocardium in Pts with LV Dysfunction:

Clinical studies. In recent years, we demonstrated that enhanced detection of ischemic but viable myocardium can be achieved by the reinjection of thallium after stress-redistribution imaging. This novel technique provides a less expensive alternative to PET for prospectively identifying viable but dysfunctional myocardium. The accuracy of this method in identifying viable myocardium has subsequently been confirmed by a large number of centers throughout the world. The reinjection method, however, is time-consuming and logistically difficult, requiring the acquisition of 3 separate image series encompassing nearly an entire day in the nuclear cardiology laboratory. An attractive alternative is to reinject 1 mCi of thallium immediately after the exercise images and acquire a modified redistribution image 3-4 hrs later representing redistribution of both the stress and the reinjected thallium doses. Applying such early thallium reinjection protocol, we found that early thallium reinjection after post-exercise imaging underestimates myocardial viability in approximately 1/4 of irreversible defects. A potential explanation for the underestimation of viability may relate to persistent reduction in regional blood flow post-exercise in a subset of pts with multivessel CAD. Therefore, our research goals are 1) to assess regional blood flow in a series of pts, using PET, at rest and post exercise, and 2) to determine whether persistent regional wall motion abnormalities following exercise represent myocardial stunning.

Histomorphologic studies of explanted hearts: In pts with impaired LV function due to ischemic cardiomyopathy, the therapeutic decision between coronary artery revascularization and cardiac transplantation can be difficult. This is particularly challenging among pts experiencing symptoms of both heart failure and ischemia. We therefore evaluated the presence and extent of viable myocardium by thallium reinjection and glucose metabolism by PET in pts with severe ischemic cardiomyopathy awaiting cardiac transplantation. After transplantation, the explanted hearts were sliced in short-axis sections and the volume fraction of myocytes from midventricular slices were studied. When matched histomorpholo-

gic, thallium and FDG regions were analyzed, we found that while there was a good correlation between % thallium uptake on redistribution and % viable myocytes by histomorphologic analysis ($r=.56$, $p<0.001$), the correlation was improved with thallium reinjection ($r=.67$, $p<0.001$) and FDG PET ($r=.67$, $p<0.001$). These data provide confirmation that the magnitude of thallium and FDG uptake correctly estimate the distribution of viable myocytes. In the future, we plan to study the non-myocyte compartment of the non-infarct region of LV, to determine whether structural remodelling of the LV contributes to the impairment of systolic and diastolic function in pts with ischemic cardiomyopathy.

Transesophageal dobutamine stress echocardiography: Dobutamine stress echocardiography is a useful method for clinical assessment of CAD pts. We recently reported on the accuracy of transesophageal echocardiography combined with dobutamine stress for the evaluation of these pts. This technique appears as ideal for assessment of pts with poor transthoracic ultrasound window and provides an excellent tool for clinical investigations based on ultrasound imaging of the myocardium.

Over the last year, we have utilized transesophageal dobutamine stress echocardiography to address specific issues in pts with hemic heart disease. Thus, we have shown that it accurately identifies stenoses of individual coronary vessels and thus permits detection of the site of coronary obstruction in atherosclerosis. We also found that the dose of dobutamine at which the initial manifestation of myocardial ischemia develops correlates with known indices of poor prognosis in pts with CAD, such as the extent of disease and the change in LV ejection fraction with exercise measured by radionuclide angiography.

We have also investigated the relation between myocyte membrane integrity (expressed as the ability of the myocardium to uptake thallium) and the contractile response to low-dose dobutamine infusion in pts with chronic CAD and LV dysfunction. We found a relationship between thallium uptake and the inotropic response to dobutamine; however, the proportion of segments showing a positive response to dobutamine was significantly lower than those with positive thallium uptake. These findings suggest that the cellular mechanisms responsible for a positive inotropic response to dobutamine require a higher degree of myocyte functional integrity than those responsible for thallium uptake.

We have also shown that transesophageal dobutamine stress echocardiography is accurate in detecting CAD in women presenting with anginal chest pain, and for the identification of pts with chest pain of non-hemic origin. In the latter pts, this methodology successfully provoked the typical chest pain experienced by the pt during daily life and demonstrated no concomitant regional wall motion abnormalities.

ANNUAL REPORT OF THE HEMATOLOGY BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
OCTOBER 1, 1993, TO SEPTEMBER 30, 1994

The major research and clinical interests of the Hematology Branch are in normal hematopoiesis and the pathogenesis, pathophysiology, and effective treatment of bone marrow failure and malignant blood cell diseases. The Branch consists of research groups in the following areas: B19 parvovirus; bone marrow failure states, including acquired and constitutional aplastic anemia and involving viral and immune studies; gene therapy, including retroviruses and adeno-associated virus vectors; and bone marrow transplantation. Included in the Hematology Branch is the 2 West clinical service and the Bone Marrow Transplant Unit. The major diseases studied include acquired aplastic anemia, Fanconi's anemia, chronic myelogenous leukemia, multiple myeloma, acute myelogenous leukemia, myelodysplasia, and congenital hemoglobinopathies. The Hematology Branch also administers an accredited and successful hematology fellowship program.

B19 PARVOVIRUS

B19 parvovirus is a human pathogen that is responsible for a number of diseases, including fifth disease in children, transient aplastic crisis in patients with sickle cell anemia and other underlying hemolytic states, a rheumatologic syndrome in adults, and pure red cell aplasia in immunosuppressed individuals. We previously identified the target cell of B19 parvovirus as the human erythroid progenitor cell. The virus' extraordinary tropism for this cell is largely mediated by its receptor, erythrocyte P antigen. A defective humoral immune response is responsible for persistent infection.

New Parvovirus Diseases

We have described a new parvovirus syndrome due to congenital infection. When fetuses are infected in mid-trimester, hydrops fetalis results. When interventional treatment with blood cell transfusions is administered either in utero or at birth, infants may be born with severe anemia, characterized morphologically as either pure red cell aplasia (Diamond-Blackfan syndrome) or a congenital dyserythropoietic state. We have shown that these infants are infected with B19 parvovirus, but low levels of virus are restricted to the bone marrow. Treatment with immunoglobulin, ordinarily effective in persistent infection, is not helpful. A secondary immune mechanism may suppress hematopoiesis in these infants. Because of our identification of P antigen as the receptor, we have also investigated paroxysmal cold hemoglobinuria, a severe hemolytic anemia of childhood that is post-viral. Preliminary studies suggest that the majority of patients with PCH suffer a post-B19 parvovirus disease, mediated either by anti-idiotype antibody formation or recognition of a neo-antigen.

Vaccine Studies

We have a Collaborative Research and Development Agreement with MedImmune to develop an effective vaccine for B19 parvovirus. In conjunction with the development of a recombinant capsid system using the baculovirus-derived antigen, we have defined multiple linear epitopes that are recognized by neutralizing antibodies against B19 parvovirus. VP1, the minor capsid protein, differs from VP2, the major capsid protein, by an additional 227 amino acids at the amino terminus. This region is highly immunogenic and it is the site of most linear neutralizing epitopes. We have now synthesized peptides of about 20 amino acids each and constructed multiple antigens on a poly-lysine backbone. Peptides from several parts of the unique region elicit neutralizing antibody responses in rabbits. A synthetic vaccine might be feasible for humans. In addition, sites of the VP1 unique region

external to the capsid have now been defined. Because the unique region of VP1 is immunogenic but not required for capsid formation, we speculated that sequences might be substituted by foreign proteins without disturbing the capsid structure. We now have succeeded in replacing the unique region with the entire hen egg white lysozyme gene. Parvovirus particles that incorporated functional lysozyme have been produced. Lysozyme is external to the capsid and retains its functional activity. These particles are now being tested for immunogenicity.

Genetic Regulation of B19 Parvovirus

We have shown that YY1, a ubiquitous bifunctional transcription regulator, binds to several sites in the terminal repeat and promoter regions of B19 parvovirus. In transfection systems, YY1 is an up regulator of B19 parvovirus RNA synthesis. An efficient purification method based on the baculovirus system has been developed for YY1. The nonstructural protein of B19 parvovirus is cytotoxic to target cells. This protein also subserves multiple functions required for parvovirus replication. The nonstructural protein contains nucleotide triphosphate bindings, and one of these sites is required for cell killing.

BONE MARROW FAILURE

Aplastic anemia has clinical and laboratory features to suggest that viruses or drugs may incite a pathophysiologic immune response. We have previously demonstrated the presence of activated cytotoxic lymphocytes that over-express gamma interferon in the blood and bone marrow of patients; these cells and lymphokines are the targets of successful immunosuppressive therapy. In Fanconi's anemia, in contrast, aplastic anemia occurs as a result of genetic lesion, probably in a protein required for DNA repair. This patient population is amenable to gene therapy.

Molecular Mechanisms for Immune Suppression of Hematopoiesis

Both gamma interferon and tumor necrosis factor alpha are over-expressed in the bone marrow of patients with aplastic anemia. Using in vitro methods, we have investigated the downstream events possibly involved in hematopoietic suppression by immune system cells. We showed that the Fas receptor is expressed on hematopoietic cells. Fas is an antigen first identified in the immune system as triggering apoptosis on binding of its ligand or a specific monoclonal antibody. For hematopoietic cells, Fas is induced in total bone marrow by positive hematopoietic growth factors, analogous to Fas expression with activation of lymphocytes. On primitive cells defined by the CD34+ antigen, Fas expression is minimal but is greatly stimulated by addition of gamma-interferon or tumor necrosis factor-alpha. Monoclonal antibody to Fas is synergistic with these two negative regulators of hematopoiesis in suppressing hematopoietic colony formation and at the level of the stem cell, as monitored by the long-term cultured initiating cell. In the CD34+ population, Fas expression and triggering is associated with apoptosis. In addition to the Fas system, we have also implicated nitric oxide as a potent downstream inhibitor of hematopoiesis. CD34+ cells contain nitric oxide synthase and are susceptible to nitric oxide inhibition.

Hematopoietic Growth Factors and Inhibitors

The proliferation and differentiation of hematopoietic cells is under both positive and negative control. Positive acting hematopoietic growth factors increase colony formation in vitro and blood counts in animals and humans. Negative regulators of hematopoiesis inhibit colony formation, are cytotoxic to bone marrow cells, decrease blood counts in treated patients, and may be pathophysiologic in diseases like aplastic anemia. A variety of expression vectors that result in production of sense or

antisense RNA have been developed and employed in the transgenic mouse system or as retroviral vectors for the transduction of murine hematopoietic stem cells. Transgenic mouse strains have been developed that express an antisense RNA complementary to the sequence of IL-3. These animals have an unusual phenotype, characterized predominantly by a neurologic syndrome of cerebellar degeneration, results that suggest a role for IL-3 in the nervous system. The mice also develop a rapidly fatal B cell lymphoma, implicating IL-3 in a critical physiologic role in the growth of early undifferentiated B lymphocytes. Similar experiments have been initiated for transforming growth factor β, a potent inhibitor of hematopoiesis. Inhibition of TGF-β production stimulates progenitor and stem cell proliferation. Finally, a retrovirus vector encoding the murine gamma interferon gene has been introduced into hematopoietic stem cells of mice. Reconstituted animals appear to have lower blood counts, consistent with the negative regulatory role of this inhibitory cytokine. Similar strategies are being employed to study the role of gp130, the common transducing component of a family of cytosine receptors that include IL-6, IL-11, LIB, oncostatin M, and CNTF. These experiments have relevance to disease pathophysiology, normal hematopoiesis, and to the development of effective strategies for improving gene transfer in vitro for human therapy.

A Putative Virus in Hepatitis/Aplasia

The immune events that characterize aplastic anemia also occur with viral infection. We have initiated a systematic survey of samples of liver and serum from patients with either hepatitis/aplasia or fulminant hepatitis, both of which we have previously shown to be non-A non-B non-C by serologic and molecular testing. However, using a variety of molecular methodologies, including gene amplification, expression cloning, and differential display, we have been unable to find evidence of a novel viral agent.

Immunosuppression for Severe Aplastic Anemia

Our study of intensive immunosuppression, consisting of antithymocyte globulin and cyclosporin in combination, has been completed by the accrual of 55 patients. We have obtained a hematologic response rate of 3 months of 71% without treatment-related mortality. Both response rate and survival are far superior to our historical control population treated with antithymocyte globulin alone. Response rates with intensive immunosuppression also were excellent both for children and severely neutropenic patients, two populations resistant to conventional immunosuppressive therapy. We are also treating patients with refractory aplastic anemia with stem cell factor, a novel cytokine that acts directly on the hematopoietic stem cell.

Epidemiology of Aplastic Anemia

A large NHLBI-sponsored epidemiologic study of aplastic anemia in Bangkok and rural Thailand will be completed this year. In addition, we have initiated an incidence study of aplastic anemia in Ho Chi Minh City, Vietnam. We will also determine whether hepatitis C virus is more prevalent in Vietnam than elsewhere in the world by serologic and molecular assays performed in our laboratory and in the Department of Transfusion Medicine.

Fanconi's Anemia

Fanconi's anemia is an autosomal recessive genetic disorder that produces aplastic anemia. Some but not all patients suffer congenital anomalies, and there is a general predisposition to malignancies. There are at least four subtypes of Fanconi's anemia as defined by complementation analysis. The gene responsible for Fanconi's anemia type C (FACC) has been molecularly cloned. We

have derived a high titer amphotropic producer clone for this gene using a retroviral packaging cell line. We have also developed a recombinant adeno-associated virus vector containing the FACC gene. Using either the retrovirus or adeno-associated virus vector, we have been able to transduce cell lines derived from patients with Fanconi's anemia type C. Cells transfected with the gene lose their sensitivity to cross-linking agents like mitomycin C and have favorable growth in vitro. We have also genetically and functionally corrected hematopoietic progenitor cells from patients with the FACC mutation. Progenitor cells were purified for CD34+ population; corrected cells form hematopoietic colonies in vitro and have normal cytogenetics. Long-term studies in the mouse have shown that the gene can be introduced into murine cells without apparent adverse effect in animals. The mechanism of action of the replaced gene appears to be shortening of the G2 phase, which is abnormally prolonged by mitomycin treatment, results consistent with FACC gene product decreasing DNA damage that accumulates prior to G2 leading to a shortened G2 phase as a result of less unrepaired DNA accumulation. The FACC gene has been successfully expressed in the baculovirus system and will be purified.

Because Fanconi's anemia is a fatal disease and no therapy other than bone marrow transplantation is available, we have initiated a protocol for gene therapy in this syndrome. A well-characterized retrovirus vector will be used. Our protocol has been approved by the Institutional Review Board and the Recombinant Advisory Committee and soon will be presented to the Food and Drug Administration. As a preliminary clinical study, we have instituted hematopoietic growth factor therapy of Fanconi's anemia. The purpose of this trial is not only to improve blood counts in these patients, but to allow harvest of the CD34+ population of cells, which contains both hematopoietic progenitors and stem cells. Peripheral blood mobilization of stem cells will be required in order to transduce the FACC gene. In the few patients so far treated, it appears feasible to mobilize hematopoietic progenitor cells from patients with bone marrow failure by this method.

The Fanconi's anemia gene transfer model has also recently been applied to the preparation of vectors for the interleukin-2 receptor gamma chain gene, in collaboration with the laboratory of Dr. Warren Leonard. The goal also here is gene therapy of patients with severe combined immunodeficiency.

Gene Transfer Into Primates

The NHLBI primate colony has now been successfully transferred to its new facility at 5 Research Court in Gaithersburg. The major purpose of this facility is to develop strategies for efficient gene transfer to primates, especially as translation of the results with murine modes has not been direct for human hematopoietic cells. The primate system allows testing of two major components of a gene therapy protocol; first, the most efficient method of expansion of the population of progenitor and stem cells in vitro and in vivo, especially the appropriate use of hematopoietic cytokines and chemotherapeutic agents; and, second, isolation of the cellular fraction responsible for reconstitution of a living animal. Because viral titers are often limiting, the highest ratio of transducing particles to target cells is desirable.

A major accomplishment during the last year has been to demonstrate that primate cells bearing the CD34 and THY1 antigens contribute to engraftment. These cells can be isolated using efficient and cost-effective magnetic separation techniques. Gene transfer into circulating leukocytes has been as high 6-10% with some vectors, evidence of successful transfer into stem cells still at low level of efficiency. Finally, great care has been taken to eliminate contamination of retroviral stocks with replication

competent helper virus.

Gene Transfer into Human Hematopoietic Cells

Our Branch pioneered a number of human gene marking protocols. We have employed retroviral supernatants to transduce marker genes into peripheral blood and bone marrow hematopoietic cells. A large proportion of progenitor cells can be transduced if the population is pre-selected for cells bearing the CD34+ antigen. In human studies, 8 patients with multiple myeloma and 5 with breast cancer have received genetically marked bone marrow and peripheral blood CD34+ cells as part of autologous transplantation and high dose chemotherapy. Nine of eleven patients have shown marked cells in bone marrow or blood or both. By using two distinct vectors to mark marrow and blood, we have demonstrated that the blood is a better target for gene transfer than bone marrow. Of patients followed more than 6 months, 3 of the 8 remain positive for the transfected gene, although the level of marked cells is very low. These results have stimulated other marking protocols in order to vary for example, the presence of growth factors or autologous marrow stromal cells, so as to improve the efficiency of gene transfer. Other gene therapy experiments include the transfer of the adenosine deaminase gene into a CD34+ blood cell population of a child with severe combined immunodeficiency. A protocol for use of a retroviral vector to transfer the glucocerobrosidase gene in two patients with Gaucher disease has been approved by the Recombinant Advisory Committee.

In laboratory studies of tissue culture systems and non-human primates, serum- free conditions have been shown to support primitive murine and human cell proliferation; in fact, the expansion of human progenitors is improved under these circumstances. Importantly, the human CD34+ cell can be transduced in the absence of serum. Other experiments have examined the role of stroma as a target for gene transduction. A murine stromal cell line has been successfully transduced with genes for interleukin-3 or interferon and then used to engraft the marrow of recipient mice.

Adeno-Associated Virus as a Vector

As described above, adeno-associated virus appears to be equivalently efficient to retrovirus vectors for the transfer of the FACC gene into human cell lines and primary hematopoietic cells. Adeno-associated virus has several advantages compared to retroviruses as a vector. The virus is not pathogenetic nor or parvoviruses in general believed to be oncogenic. For adeno-associated virus, there is cell culture data convincingly indicate that the virus can persist by integration into the host genome, a prerequisite for an effective transducing agent. For one cell line, a specific integration site in chromosome 19 has been described. Adeno-associated virus does have a limited genome, and the size of the packaged insert is restricted. Furthermore, a packaging cell line has not been developed, and virus production requires a cumbersome methodology which has not yielded high titers of infectious virus obtained.

Our culture system for the production of adeno-associated virus requires a source of nonstructural protein or rep as well as super-infection with adenovirus. We have previously shown that an adeno-associated virus recombinant that incorporates a human globin gene efficiently transduced human bone marrow progenitor cells. A relatively large proportion of burst forming cells, early erythroid progenitors, were transduced, and the levels of globin protein produced were substantial. The globin gene experiments required the selective gene for neomycin resistance. Recently, an adeno-associated virus vector was constructed to contain a gene for CD4, the receptor for HIV-1 and an important T cell surface molecule. This vector was used to transfect a cell line, which was cultured in the absence of

selective pressure. Under these circumstances, an extremely high degree of efficiency of transduction could be documented at 48 hours, with virtually all cells bearing CD4 as determined by flow cytometry. By comparison to growth under selective pressure at two weeks, approximately 100-fold higher transduction was observed transiently than permanently. Evidence for integration by Southern analysis was obtained, although multiple integration sites are probably utilized. The CD4 transfection system offered a convenient and rapid method to access viral titers and efficiency of transduction.

The cellular receptor for adeno-associated virus is unknown. Based on our prior experience with the identification of the receptor for B19 parvovirus, we have initiated studies to identify the receptor for adeno-associated virus. To date, we have successfully labeled virus with biotin and demonstrated specific binding by flow cytometry using HeLa cells. These studies should allow definition of the full range of tissue tropism for adeno-associated virus.

BONE MARROW TRANSPLANTATION

The new Bone Marrow Transplant Unit opened in 1993 and the first allogeneic transplant was performed in September. To date, we have performed a total of 8 such transplants for chronic myelogenous leukemia and myelodysplastic syndromes. Five of six patients transplanted from fully matched donors have survived without leukemia. Three patients have died, two following failed engraftment from partially matched donors and one from graft-versus-host disease and infection.

The major thrust of current clinical protocols is to achieve a practical separation of graft-versus-host disease from graft-versus-leukemia effects. Observations in mice have suggested that the delayed addition of lymphocytes allowed engraftment, including immune reconstitution, with decreased graft-versus-host-disease, and this protocol has been applied to humans. Bone marrow is depleted of T cells prior to infusion into patients, and lymphocytes from the donor are infused into the recipient at 30 and 45 days after transplant. A sensitive helper T-lymphocyte precursor frequency assay has been used to monitor donor response to leukemia before and after lymphocyte addition.

In the laboratory, major efforts have been directed at defining leukemia-related alloantigens for the depletion of graft-versus-host reactivity. The K562 cell line, which lacks HLA surface antigens, has been transfected with genes for several HLA antigens. Reactive T lymphocyte clones which share HLA identity with the transfected K562 cell line can be produced against these transfectents; the specificity of the antigens expressed will then be characterized. A similar approach is employed to raise alloreactive T lymphocyte clones to fresh leukemia cells. In collaboration with Dr. Steven Rosenberg, this system has been applied to melanoma antigen identification, and T cell clones will be used to probe a cDNA library made from K562 cells to identify protein sequences. Both the nature and specificity of immunodominant peptides important in graft-versus-host disease and the graft-versus-leukemia effect can be determined. In order to deplete donor lymphocytes which react with non-leukemic host cells, the IL-2 receptor, a marker of lymphocyte activation, is being targeted by a specific anti-CD25 antibody conjugated to a toxin. Lymphocyte frequency can be quantitated before and after this method. Graft-versus-host disease depleted lymphocytes may be clinically useful in the treatment of leukemia, even independent of bone marrow transplantation.

ANNUAL REPORT OF THE HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October I, I993 through September 30, I994

The Hypertension-Endocrine Branch pursues studies into the mechanisms of blood pressure regulation and the causes and treatment of hypertension and its complications. In the last year our activities have been concentrated in three areas: I) vasoactive substances, 2) cardiac hypertrophy, and 3) pheochromocytoma.

I. Vasoactive substances. Congestive heart failure (CHF) is a frequent complication of hypertension. It is a pathologic condition characterized by avid sodium retention and edema formation. The exact mechanisms underlying the exaggerated sodium reabsorption in CHF have not been clarified. Several mechanisms have been implicated, including the renin-angiotensin-aldosterone system, the sympathetic nervous system, and attenuated responses to atrial natriuretic peptide (ANP), an important hormone in the regulation of sodium and water homeostasis. Neutral endopeptidase (NEP) is a widely distributed enzyme found predominantly in the kidneys and lungs and involved in the degradation of ANP and other peptides, including bradykinin, enkephalins and endothelins. We sought to determine the importance of NEP in CHF by 1) use of an NEP inhibitor, SQ-28,603, and 2) defining the concentration and activity of NEP in the lungs and kidneys of rats with either compensated or decompensated CHF. We performed the studies in a model of high output CHF produced in rats by means of an aortocaval fistula (ACF), distal to the renal arteries. In sham-operated control rats, we found that NEP-I, in a dose of 40 mg/kg I.V., caused marked increases in urine output and in both absolute and fractional sodium excretion. Infusion of the same dose of NEP-I into rats with compensated CHF also induced a remarkable natriuresis and diuresis. However, the response was markedly blunted in rats with decompensated CHF. Quantitative reverse transcription-polymerase chain reaction (RT-PCR, 2I cycles), Western blot analysis using polyclonal antibodies against NEP, and visualization of NEP activity (using a gel in which SDS polyacrylamide was co-polymerized with gelatin) revealed that NEP-mRNA, NEP-immunoreactivity and NEP-activity in the kidneys were unaltered during CHF. However, NEP-mRNA, NEP-immunoreactivity and NEP-activity were significantly lower in lungs of decompensated rats than in either compensated rats or control animals. This is significant because it demonstrates that inhibition of NEP induces significant diuresis and natriuresis in mild, but not in severe, CHF. Renal NEP activity is not altered even in severe heart failure, but pulmonary NEP activity is markedly reduced. These findings indicate that the blunted response to ANP in CHF is not due to changes in endogenous renal NEP-activity since the latter do not change. In addition, the effect is not due to changes in pulmonary NEP-activity since the decrease in the latter would have the opposite effect and yield higher levels of circulating ANP.

Endothelin (ET) is an extremely powerful vasoconstrictor peptide, produced in vascular endothelium. ET is derived from a 38 amino acid precursor peptide, Big ET (BET). Reports of studies of cultured endothelial cells had indicated that insulin was a strong stimulant for ET release. Therefore, we studied the effects of hyperinsulinemia and sodium intake on both plasma and urinary levels of ET in normal subjects via use of an insulin clamp, which produces a controlled safe level of hyperinsulinemia. The insulin-clamp study was performed three times with the patient on different levels of sodium intake. We have studied II normotensive subjects and found that hyperinsulinemia increased urinary excretion of ET consistently, but not significantly. The largest increase, 36%, was observed in subjects on a high sodium diet.

Increases in urinary excretion of ET were always accompanied by significant increases in urinary flow. There was a strong correlation between urinary excretion of ET and urinary flow (r=0.8, p=0.001), which raises the possibility that the rate of ET excretion is a passive phenomenon related to "washout". Twenty-four hour UETV in subjects on a normal sodium intake was 197±19 ng/d, and it increased to 225 ±40 ng/d (p=NS) on a high sodium intake. There was also a correlation between UETV and sodium excretion (r=0.53, p=0.01). In contrast, neither insulin infusion nor changes in sodium intake significantly affected plasma levels of either ET or ANP. This would indicate that in man insulin has only a mild effect on urinary ET production and release, while urinary flow appears to have a much greater effect and sodium intake has only a minor effect. The clinical study will be redesigned to better test the effects of fluid loading and urinary flow rate on ET excretion.

II. Cardiac hypertrophy (CH) is a major risk factor for cardiac morbidity and mortality. In our studies, we have attempted to: 1) identify genes, the expression of which is universally altered in CH, irrespective of the model, and 2) examine the underlying mechanisms and the functional significance of known biochemical changes in the heart that are characteristic of specific models of CH. CH was induced in rats by both pharmacological and surgical means, including DOCA-salt hypertension, hypertension due to inhibition of nitric oxide synthase, spontaneously hypertensive rats, rats with high-output congestive heart failure induced by an aortocaval fistula, and either repeated injections or continuous infusions of phenylephrine (PE), isoproterenol (Iso), or angiotensin II (AII). The hearts were excised at different stages either after the initial intervention or after cessation of the hypertrophic stimulus, and their weight, macroscopic and microscopic structure, and molecular and biochemical markers were examined. We have found that two distinct patterns of CH are induced by different hormonal stimuli. The first, exemplified by excessive exposure to a beta-adrenergic agonist, i.e., Iso, is characterized by rapid onset, large maximal response, reversibility after cessation of the stimulus, and a decrease in the extent of the response with age. The extent of CH induced by this stimulus is equal in males and females. The second pattern of CH was induced by excessive exposure to either an alpha-adrenergic agent, i.e., PE, or to AII and is characterized by slow onset, small maximal response, and very slow regression (if any) of CH after cessation of the stimulus. The extent of the hypertrophic response does not decrease with age and is greater in males than in females. Biochemically the latter, but not the former, pattern of hypertrophy is characterized by a decrease in the relative levels of mRNA encoding calcium-dependent sarcoplasmic reticular ATPase (CA-SR-ATPase). In addition, CH of the second pattern is characterized by an increased ratio of beta-myosin heavy chain (beta-MHC) to alpha-MHC, whereas the first pattern is characterized by a decrease in this ratio. The change in MHC-isoforms induced by PE occurs equally in animals that respond to PE with CH and in those that do not, indicating that the MHC change and CH are independent adaptive processes. The motility of labeled actin filaments on myosin extracted from hearts of Phe-treated adult rats was normal as determined by the "in vitro motility assay." In contrast, actin motility on myosin extracted from hearts of adult rats treated with Iso exhibited an increased cycling rate. These data clearly show that at least two different patterns of CH result from different types of stimuli. We have also shown that the initiation and the maintenance of CH are independent and respond to different stimuli.

The ratio between mRNA encoding for alpha- and beta-MHC was determined by competitive PCR. We found that changes in the ratio between these proteins are not paralleled by changes at the RNA level, indicating significant post-transcriptional regulation on the expression of these proteins by adrenergic stimuli. However, a different regulatory process

predominates during regression from CH, i.e., after removal of the initiating stimulus. During regression from PE-induced CH, the ratio between MHC's returns within days from a large excess of beta-MHC to the normal for the animal's age. During this regression period, the ratio between beta- and alpha-MHC-mRNA decreases dramatically so that beta-MHC-mRNA is barely detectible. The reverse occurs during regression of Iso-induced CH. This suggests mainly transcriptional control over MHC expression during the reversal of the effect of the drugs. Thus, there is primary post-transcriptional and secondary transcriptional regulation of the expression of MHCs in response to adrenergic stimuli.

Using subtractive hybridization, we cloned four genes that were specifically expressed in hearts from both patterns of CH, but not in normal hearts. Localization of the expression of these genes in the hypertrophied hearts by means of in situ hybridization revealed that all four of them are expressed in cardiac connective tissue and not in myocytes. Therefore, these genes are probably indicative of dividing connective tissue cells and are not characteristic of the hypertrophic process in myocytes. We are now in the process of cloning genes that are specifically expressed in myocytes of the hypertrophied hearts by separating myocytes from other cells before RNA extraction. We will continue this work because of the importance of identifying the early events that trigger CH.

III. Pheochromocytoma. We have continued our efforts to improve the diagnosis, localization, and treatment of pheochromocytoma and paraganglioma. In collaboration with the Clinical Neuroscience Branch, NINDS, we have evaluated the clinical usefulness of a new technique for measuring metanephrines, i.e., metanephrine (MN) and normetanephrine (NMN) in blood by means of liquid chromatography and electrochemical detection. We have applied this technique, along with measurements of plasma catecholamines and urinary metanephrines, to 42 patients with pheochromocytoma, 34 patients with essential hypertension, and 44 normotensive control subjects. We found that a pheochromocytoma/paraganglioma resulted in considerably larger increases in plasma metanephrines than in catecholamines. In normal volunteers and patients with hypertension, plasma concentrations of both NMN and MN form a well-defined range. Plasma NMN concentrations were increased in all patients with pheochromocytoma, whereas MN concentrations were increased in only some of the patients. No patient with a pheochromocytoma had plasma concentrations of NMN and MN that both yielded false negative results, i.e., 100% sensitivity. If the diagnosis of pheochromocytoma was based on plasma catecholamines, there were six false negatives, i.e., 86% sensitivity, while for urinary metanephrines there were three false negatives, i.e., 92% sensitivity. False positive rates were 12% for both plasma metanephrines and catecholamines. Thus, for equal sensitivities, plasma metanephrines provided significantly better specificity than did plasma catecholamines. The finding of normal plasma metanephrines virtually excludes the diagnosis of pheochromocytoma, whereas the finding of normal plasma catecholamines or normal urinary metanephrines does not. Therefore, measurement of plasma metanephrines provides the most sensitive, single clinical test yet described for the diagnosis of pheochromocytoma. We will continue to evaluate the usefulness of this new assay in the diagnosis of pheochromocytoma.

MOLECULAR DISEASE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

The major lipids, cholesterol and triglycerides, are transported in the blood by lipoproteins. Lipoproteins contain both lipids including cholesterol, phospholipids and triglycerides as well as proteins, designated apolipoproteins (apo). The overall objective of the combined basic and clinical research programs of the Molecular Disease Branch is the elucidation of the role of plasma lipoproteins in lipid transport in normal individuals and in patients with elevated plasma lipid levels who are at risk for the development of early heart disease or other diseases including pancreatitis. A major focus of research in the Branch is the analysis of the physiological role of apolipoproteins and lipoproteins in cholesterol and triglyceride transport, and the elucidation of the pathophysiological mechanisms involved in the regulation of lipoprotein biosynthesis, transport, and catabolism in patients with disorders of lipoprotein metabolism and atherosclerosis. Studies are also conducted to establish the genetic defects in patients with elevated blood lipids in order to establish improved methods for detection of individuals at risk for heart disease or pancreatitis. The ultimate goal of this research program is the development of new methods for diagnosis as well as the advancement of more definitive approaches to therapy including gene therapy for the genetic disorders of lipid metabolism.

Regulation of Lipoprotein and Hepatic Lipases

Lipoprotein lipase (LPL) and hepatic lipase (HL) are attached to the capillary endothelium of peripheral tissues and the liver, respectively. LPL is the principal enzyme responsible for the hydrolysis of plasma triglycerides. HL functions primarily as a phospholipase and is a key enzyme in the conversions of IDL to LDL and HDL_2 to HDL_3. An understanding of the structure and function of LPL and HL are critical for our understanding of the biological function of these key enzymes in lipid and lipoprotein metabolism in normal subjects and in patients with dyslipoproteinemias. LPL, HL, and pancreatic lipase (PL) have a high degree of structural homology and form a lipase gene family. The crystalline structure of PL has been elucidated and used as a molecular model for the lipase gene family. Studies in the Branch have utilized site-directed mutagenesis and construction of chimeric enzymes to gain insight into the functional domains of LPL and HL.

LPL contains a "lid" or loop of 22 amino acids from cysteine residue 216 to cysteine residue 239 which has been proposed to play an important role in modulating the access of lipid substrates to the catalytic site of the enzyme. Two amphipathic helices are present within this lid. To gain further insight into the structural basis for the different substrate specificities of LPL and HL both in vitro and in vivo studies were undertaken to generate mutant lipases using site-directed mutagenesis as well as recombinant adenoviral vectors. Disruption of the 2 amphipathic helices present in the lipase lids results in loss of activity using either triolein or phospholipid (PL) substrates while esterase activity against the short-chained water soluble substrate tributyrin is preserved. Chimeric lipases containing the LPL backbone with the HL lid, the HL backbone with the LPL lid and the amino terminus of LPL with the carboxyl-terminus of HL had markedly different abilities to hydrolyze PL substrates. The presence of the HL lid augmented PL hydrolysis whereas the LPL lid enhanced triglyceride hydrolysis. These in vitro studies have now been extended in vivo using recombinant adenovirus expressing either native HL or HL with the LPL lid. These constructs were injected in HL-deficient mice who have elevated plasma levels of total cholesterol and phospholipids. Animals injected with the native HL virus had a significant decrease in plasma PL and a marked change in lipoprotein profile; however, those injected with HL with the LPL lid had no change in plasma PL levels. These studies indicate that exchange of the HL with the LPL lid modifies the ability of the lipase, in vivo, to hydrolyze PL and thus, established an important role of the lipase lid in mediating lipase substrate specificity. Based on these combined results it has been concluded that the two amphipathic helices in both LPL and HP are essential for phospholipid as well as triglyceride hydrolysis. The HL lid increases phospholipid hydrolysis whereas the LPL lid enhances triglyceride hydrolysis. These studies indicated that the lid may play a pivotal role in determining the substrate specificity of both LPL and HL.

CLINICAL DISORDERS OF LIPOPROTEIN METABOLISM

Elevated blood levels of LDL and reduced levels of HDL have been well established as important

risk factors for the development of early heart disease. Elevated levels of plasma triglycerides have been associated with both pancreatitis and early heart disease. A major focus of research in the Branch is a systematic analysis of triglyceride rich lipoproteins and HDL in normal subjects and in patients with both elevated and reduced levels of plasma triglycerides and HDL.

A. HDL metabolism

Plasma HDL are heterogeneous and different lipoprotein particles within HDL have been proposed to have separate physiological functions. Two important classes of lipoprotein particles within HDL are particles which contain both apoA-I and apoA-II (LpA-I:A-II), and particles which contain only apoA-I (LpA-I). LpA-I:A-II is formed by the addition of apoA-II to the LpA-I particle. LpA-I particles have been proposed to be the important anti-atherogenic particles within HDL which are involved in the removal of excess cholesterol from cells by a process which has been termed reverse cholesterol transport. LpA-I:A-II particles appear to offer little protection against the development of early heart disease. We have performed a series of detailed studies on LpA-I and LpA-I:A-II metabolism in normal subjects and subjects with reduced and elevated levels of HDL cholesterol. Detailed kinetic studies in normal volunteers established that LpA-I was catabolized at a significantly faster rate than LpA-I:A-II.

Three subclasses of LpA-I are present in human plasma. These three subclasses designated Large, Medium, and Small have different lipid and apolipoprotein composition as well as concentrations of CETP and LCAT activities indicating the important heterogeneity of the LpA-I particles. The metabolism of the three LpA-I particles was investigated in control subjects. The catabolism of the Small LpA-I was the fastest of the three LpA-I particles clearly establishing that the catabolism of LpA-I is critically dependent on the size of the LpA-I particle.

To gain further insight into the metabolism of LpA-I and LpA-I:A-II, kinetic studies were performed in fifty normolipidemic subjects and the associations with lipids and apolipoprotein levels determined. Triglyceride levels inversely correlated with only LpA-I. ApoA-I levels were most strongly correlated with the catabolic rate. ApoA-II levels were only correlated with the rate of apoA-II synthesis. Plasma LpA-I levels are correlated with the catabolism of both apoA-I and apoA-II while LpA-I:A-II levels were correlated with the synthesis rates of apoA-I and apoA-II. Based on multiple regression analysis the production rate of apoA-II is the only independent kinetic parameter determining the plasma LpA-I levels.

Plasma HDL cholesterol is inversely correlated with the development of premature cardiovascular disease (CVD). HDL screening will identify individuals with low HDL that require evaluation for increased CVD risk. Not all individuals with low HDL are at increased CVD risk. Genetic diseases characterized by low levels of HDL not associated with markedly increased CVD risk include LCAT deficiency, Fish Eye disease, and Tangier disease. Recently we identified kindreds with familial hypoalphalipoproteinemia (F Hypoalpha) with HDL from 7 to 15 mg/dl and no premature CVD. Kinetic studies in five F Hypoalpha probands without CVD revealed that both LpA-I and LpA-I:A-II are rapidly catabolized leading to low HDL levels. Catabolism of LpA-I:A-II is slightly faster than LpA-I leading to a normal or increased ratio of plasma LpA-I/LpA-I:A-II.

The combined results from these studies have clearly shown that hypoalphalipoproteinemia is heterogeneous and that not all patients with low HDL have an increased risk of premature CVD. Metabolic studies have shown that the metabolism of LpA-I and LpA-I:A-II are effected independently in patients with low HDL levels. These data will provide new approaches to the identification of which individuals with low HDL are at increased risk of premature CVD.

B. Familial Hypercholesterolemia

Patients homozygous for familial hypercholesterolemia (FH) manifest profound hypercholesterolemia, cutaneous cholesterol deposits termed xanthomas, and cholesterol deposition in a variety of tissues including the eye, tendons, and inside the arterial vessels. These patients experience accelerated atherosclerosis and can manifest symptomatic cardiovascular disease from the ages of 2-30 years, and many die before the age of 20. The molecular defect in FH is a defect in the LDL receptor pathway. We have previously demonstrated that the degree of LDL receptor dysfunction on cultured skin fibroblasts from these patients highly correlates with the concentrations of LDL cholesterol present in their circulation. In recent studies the post-heparin infusion concentration of

lipoprotein lipase mass was shown to correlate with the severity of atherosclerosis in homozygous FH. A variety of therapies have been used to reduce the LDL cholesterol concentrations in these patients including diet, combination hypolipidemic drug therapy, portacaval shunting of the liver, plasma exchange, LDL apheresis, liver transplantation, and most recently, adenoviral gene therapy. For the past several years we have prospectively evaluated the rate of progression of atherosclerosis by both invasive and noninvasive techniques in these patients. The assessment of the extent and severity of both cholesterol deposition into tissues and atherosclerosis has been determined using computerized axial tomography. This noninvasive test led to a new concept in atherosclerotic cardiovascular disease risk assessment, the cholesterol-years risk score. These findings in this inborn error in lipoprotein metabolism may have theoretical and practical implications for individuals with more common forms of atherosclerosis.

C. Clinical Studies on Lp(a)

Lp(a) is an LDL-like lipoprotein which contains a unique apolipoprotein designated apo(a) which has a high structural homology with plasminogen. Increased plasma levels of Lp(a) are associated with an increased risk of premature cardiovascular disease. Apo(a) is polymorphic and there is a series of isoforms of the apolipoprotein in the plasma ranging in molecular weight from 400K to 600K. The size and plasma levels of Lp(a) are genetically determined. Metabolic studies in subjects with different isoforms and plasma levels of Lp(a) established that the size of the apo(a) isoform does not effect Lp(a) catabolic rate but rather the rate of synthesis of the individual isoform. The larger the size of isoform the lower the rate of synthesis. These results have established that the synthesis of apo(a) is the major determinant of the plasma levels of Lp(a) and that variations in rates of catabolism does not play a major role in determining plasma levels of Lp(a).

The pathway for catabolism for Lp(a) has not been established and it has been controversial if the LDL receptor is important in Lp(a) metabolism. The role of the LDL receptor in Lp(a) catabolism has been analyzed using Lp(a) kinetics in patients with familial hypercholesterolemia (FH) who lack the LDL receptor. Lp(a) levels are elevated in FH and it has been proposed that this is due to delayed catabolism secondary to the LDL receptor defect. Studies on four FH patients revealed that the catabolism of radiolabeled Lp(a) was similar in control subjects and the four FH patients indicating that the LDL receptor does not play a major role in the catabolism of Lp(a). The increased plasma levels of Lp(a) are due to increased production. In addition, it was also demonstrated for the first time that there was conversion of Lp(a) to LDL in vivo indicating that some of the Lp(a) is converted to LDL in the normal metabolic pathway of Lp(a) metabolism.

Molecular Defects in the Genetic Dyslipoproteinemias

The elucidation of the molecular defects in patients with genetic dyslipoproteinemias provides the unique opportunity to extend our knowledge of the role of specific apolipoproteins, enzymes, and receptors in lipoprotein metabolism. In addition it facilitates the development of new diagnostic methods to identified those individuals at risk for the development of premature cardiovascular disease prior to the onset of significant disease. The determination of the specific molecular defects in the genetic dyslipoproteinemias is a necessary prerequisite to the application of future treatment of these patients with gene therapy.

A. Cholesterol ester transfer protein (CETP) Deficiency

CETP is present in normal plasma primarily in HDL on the LpA-I particles. CETP functions in lipoprotein metabolism in the exchange of cholesteryl esters and triglycerides between HDL and the apoB containing lipoproteins VLDL, IDL, and LDL. A deficiency of CETP is associated with hyperalphalipoproteinemia. All of the reported cases of CETP deficiency have been identified in Japan and virtually all patients have the same splice site mutation in the CETP gene. The plasma lipoproteins in CETP are quite unique and are characterized by markedly elevated plasma levels of HDL particles, reduced LDL, and triglyceride enrichment of HDL as well as LDL. The elevated levels of HDL have been proposed to be protective and the subjects with CETP deficiency have been reported to have a longevity syndrome.

We have identified a new molecular defect responsible for CETP deficiency in a proband

presenting with TC, HDL-C and apoA-I levels of 300, 236 and 233 mg/dl, respectively and total absence of plasma CETP activity and mass. Sequence analysis of the patients's gene revealed that the splice donor consensus GT was substituted by GG in intron 10 and by AT in intron 14. Restriction digestion using NdeI and MaeIII established that the patient was a compound heterozygote for both gene defects. Sequencing of RT-PCR amplified DNA from macrophage RNA demonstrated abnormal splicing with deletion of exon 10 as well as alternative splicing at a native AG site located 31 nucleotides 5' of the normal splice acceptor in intron 13. This defect results in the insertion of a 31 bp fragment between exon 13 and exon 14 as well as the introduction of an in frame stop codon. The presence of abnormally spliced mRNA was further confirmed by amplification of patient RT-PCR DNA using CETP specific primers thus establishing the functional significance of these defects. These studies provide new information on the heterogeneity of molecular defects leading to CETP deficiency.

B. Lecithin:cholesterol Acyltransferase Deficiency

Lecithin cholesterol:acyltransferase (LCAT) is a plasma enzyme present primarily on HDL as well as LDL that is responsible for the esterification of virtually all free plasma cholesterol. Cholesteryl esters formed by LCAT are incorporated into the core of HDL and LDL particles. LCAT has been suggested to play a major role in reverse cholesterol transport with esterification of the cholesterol removed from cells and the transfer of the cholesteryl esters into the core of the lipoprotein particle for transport in plasma.

Patients with functional defects in LCAT present with two strikingly different clinical features. In Classical LCAT deficiency the patients have severe HDL deficiency, hemolytic anemia, cloudy corneas, and progressive renal disease. In a separate clinical syndrome, Fish Eye Disease (FED), the patients have HDL deficiency, severe cloudy corneas, but no renal disease or hemolytic anemia. During the last two years we have systematically determined the molecular defects in the LCAT gene in probands with both Classical LCAT deficiency and FED. These studies have been extended to the analysis of a novel mutation in the LCAT gene of a French patient presenting with corneal opacities and HDL-C levels of less than 10 mg/dl. DNA sequencing and RFLP analysis revealed that the patient is a compound heterozygote for a T to C and --- to ---mutation resulting in the substitution of ....to ..... and --- to --- , respectively. In vitro expression of the two mutant LCAT established the functional significance of both gene defects. Based on the combined results from these studies we have determined that the reason for the difference in the two clinical syndromes associated with LCAT deficiency is the residual activity of the mutant LCAT enzyme. Approximately 10 to 20% residual activity of the mutant LCAT enzyme appears to be sufficient activity to prevent the renal disease and hemolytic anemia.

Transgenic Models of Lipoprotein Metabolism

The development of the methodology for overexpressing specific genes in transgenic animals provides the unique opportunity to study the structure and function of genes important in lipoprotein metabolism and the development of atherosclerosis. Transgenic mice and rabbits overexpressing several different genes have been developed in the Branch.

A. Transgenic Mice Models

In order to evaluate the role of LCAT in HDL metabolism, a 6.2 kb fragment of the human LCAT (hLCAT) gene was utilized to develop three separate transgenic mouse lines overexpressing hLCAT at plasma levels 10,14 and 100 fold higher than control mice. LCAT activity in 45 heterozygous and homozygous mice ranged from 582 ± 92 to 3695 ± 340 nmol/ml/h (NL = 31 ± 4 nmol/ml/h). Northern blot hybridization analysis demonstrated tissue specific expression of hLCAT in mouse liver. Compared to 24 age and sex-matched siblings, transgenic mice had elevated plasma TC (133-237% of NL), CE (141-267% of NL) and HDL-C (123-209% of NL) but similar plasma levels of triglycerides, PL, B-containing lipoproteins, apoA-I and apoA-II. FPLC analysis of hLCAT transgenic mouse plasma revealed larger sized HDL particles enriched in CE and PL.

To gain insight into the effects of LCAT on diet induced hyperlipidemia age/sex matched LCAT transgenic and control animals were placed on a high cholesterol-fat diet for 21 d to investigate the potential role of LCAT in modulating dietary responses. Pre-diet lipid values (mg/dl) in controls were

TC = 97 ± 11, HDL = 72 ± 10 and in transgenics were TC = 121 ± 16, HDL-C = 84 ± 17. Post-diet lipid values in controls were TC = 290 ± 55, HDL = 85 ± 15, and in transgenics were TC = 313 ± 83, HDL = 115 ± 27. Thus, on the high cholesterol-fat diet transgenic mice had significantly higher ($p < 0.05$) HDL-C as well as reduced TC/HDL ratios than controls, without differences in TG, PL, CE, LCAT mass and activity. FPLC analysis of transgenic mouse plasma revealed significant increases in HDL-C, CE and PL with reciprocal decreases in IDL/LDL-C, CE and PL. These results indicate that LCAT overexpression reduced the dietary induced increase in atherogenic IDL/LDL particles and thus provide protection against diet induced atherosclerosis in this mouse model.

The function of apoA-II in lipoprotein metabolism was assessed by performing in vivo 125I-apoA-I and 131I-apoA-II kinetic studies in mice overexpressing mouse apoA-II and age matched controls. Plasma lipid and apolipoprotein levels (mg/dl) in the mice overexpressing apoA-II were TC = 267-335, Tg = 70-344, apoA-I = 88-112 and apoA-II = 22-55 while controls averaged TC = 87 ± 13, Tg = 54 ± 8, apoA-I = 88 ± 12 and apoA-II = 13 ± 0.5. ApoA-I was catabolized slower than apoA-II in control mice with fractional catabolic rates (FCR) of 1.14 ± .06 d-1 and 1.57 ± .12 d-1, respectively. In apoA-II mice overexpressing apoA-II , apoA-I and apoA-II were catabolized slower (FCR = .96 ± .06 d-1, production rate [PR] = .010mg/dl and FCR = 1.25 ± .04 d-1, PR = .005mg/dl, respectively). With progressive increases in plasma apoA-II levels there was a parallel increase in plasma triglycerides and IDL sized lipoproteins containing two major lipoproteins particles, LpB and LpA-II:E. The triglyceride-rich lipoproteins had virtually no apoE by FPLC analysis.

These studies indicate that in marked contrast to human studies, apoA-I catabolism is slower than apoA-II in the mouse. Catabolism of apoA-I and apoA-II was slower in the apoA-II overexpressors compared to the controls. The increased HDL levels in the mice overexpressing apoA-II are due to both an increased synthesis and decreased catabolism of HDL.. With progressive increases in plasma apoA-II levels there was a parallel increase in plasma triglyceride and IDL sized lipoproteins containing two major lipoprotein particles, LpB and LpA-II:E.

B. Transgenic rabbit model

A transgenic rabbit program has been developed to systematically investigate the impact of genes relevant to HDL as well as LDL metabolism, reverse cholesterol transport, and atherosclerosis. The methods required for the rabbit transgenic program including the superovulation strategies, mating and breeding techniques, and DNA as well as specific protein assays have all been developed. Initial studies were undertaken to examine the effect of overexpression of apoA-I and increased HDL levels in an rabbit model with a partial deletion of a portion of the LDL receptor binding domain leads to both profound hyperlipidemia and atherosclerosis. This strain of rabbit, termed the Watanabe Heritable Hyperlipoproteinemic (WHHL) rabbit, provides a means to test a variety of hypotheses relevant to lipoprotein metabolism and atherosclerosis. Initial studies were required to determine the endocrinologic basis for infertility in WHHL rabbits including the characterization of aberrant corpus luteal steroidogenesis. After modification of the superovulation strategies, 14 founder transgenic rabbits expressing apolipoprotein A-I were developed. The impact of overexpression of apolipoprotein A-I on both lipoprotein metabolism and the ability of HDL to prevent atherosclerotic cardiovascular disease is currently underway.

The effect of overexpression of LCAT on plasma lipoprotein metabolism in the rabbit has been initiated. Two founder transgenic rabbits overexpressing LCAT with marked elevations in HDL cholesterol have been identified. The HDL and the changes in lipoprotein metabolism resulting from LCAT overexpression are currently under investigation. The establishment of a transgenic rabbit program permits the direct testing of the reverse cholesterol transport hypothesis, a central concept in cardiovascular research.

Gene Therapy for the Genetic Dyslipoproteinemias

The ultimate correction of the molecular defects in patients with the genetic dyslipoproteinemias will be greatly facilitated by the development of gene therapy technology. In order to establish the safety and efficacy of this approach we have initiated a gene therapy program for the correction of gene defects in mice in which the function a specific gene has been inactivated by the use of homologous recombination. These gene knock out mice models provides a unique opportunity to study the

correction of gene defects by gene therapy.

A. ApoE Deficient Mice

Apolipoprotein E (apoE) is a 299 amino acid protein present in VLDL, IDL and HDL that plays a major role in the metabolism of plasma lipoproteins. ApoE is a major ligand for the LDL and remnant receptors and thus, necessary for the normal clearance of remnant particles from the circulation. Patients with a functional deficiency of apoE can develop Type III hyperlipoproteinemia and premature atherosclerosis. Recently, apoE deficient mice generated by homologous recombination have been developed with marked hypercholesterolemia and spontaneous vascular lesions. These animals are a useful model for evaluating the potential for gene therapy. We have generated a recombinant adenoviral vector containing human apoE cDNA (rAdV) for injection of apoE deficient mice. The lipoprotein profile of the apoE deficient mice included TC 609 ± 108mg/dl, TG 101 ± 50mg/dl, and cholesterol-rich VLDL/IDL present on FPLC. After IV infusion of the apoE rADV, apoE-deficient mice had peak (day 6) plasma human apoE levels of 2.3 mg/dl and 648 mg/dl. Western blot analysis demonstrated the expression of a normal sized human-apoE. Expression of these two different levels of apoE in plasma resulted in markedly different lipoprotein changes. Mice achieving physiologic apoE levels (2.3 mg/dl) normalized their lipids (TC = 109 ± 19mg/dl, TG = 56 ± 29mg/dl) at days 4-8; FPLC was normalized with loss of all VLDL/IDL and generation of HDL. Animals with 649 mg/dl, a 200 fold increase in apoE, had a biphasic lipid response with initial decrease in TC to 230mg/dl but increase in TG to 652 mg/dl; FPLC shifted from cholesterol-rich VLDL/IDL to Tg-rich LDL remnants. By 8-12d apoE (<10mg/dl) and triglycerides (66 ± 40mg/dl) decreased and FPLC revealed a normal HDL profile. ApoE expression and normal plasma lipids were maintained for a period of 4 weeks after virus injection. These studies indicate successful physiologic replacement (2.3mg/dl) as well as marked overexpression (>500mg/dl) of human apoE in apoE deficient mice using recombinant adenovirus. Physiologic levels of human apoE normalized plasma lipids whereas apoE overexpression resulted in transient formation of TG-rich remnants possibly due to high apoE levels blocking receptor mediated remnant clearance. Successful replacement of apoE in apoE-def mice demonstrates the feasibility of gene therapy in human apolipoprotein deficiencies.

B. Hepatic Lipase Deficiency

Hepatic lipase (HL) is an endothelial bound enzyme which mediates the hydrolysis of triglycerides and phospholipids present in IDL and HDL and thus, plays a central role in normal lipoprotein metabolism. Patients with HL deficiency appear to be at increased risk for atherosclerosis. In order to evaluate the feasibility of replacing an endothelial bound lipolytic enzyme in an animal model for HL deficiency, we have generated a recombinant adenovirus vector containing the human HL cDNA and the CMV-promoter (rAdV). HL deficient mice were injected IV with HL rAdV. Preinfusion lipid values (mg/dl) in the HL deficient mice included TC 146 ± 29, PL 283 ± 56, TG 70 ± 34, CE 80 ± 41, FC 66 ± 33. HL deficient mice showed increased TC (NL = 101 ± 8, $p<0.001$), PL (NL = 181 ± 15, $p<0.001$), and FC (NL = 35 ± 3, $p<0.005$) when compared to age/sex matched normal mice. The FPLC profile of HL deficient mice demonstrated a marked increase in HDL-C and PL content relative to control mice. Immunoblot analysis of mouse post-heparin plasma 4 days post-infusion of HL lipase rAdV demonstrated the presence of normal sized HL lipase. Virtually all expressed HL was detected in post-heparin plasma indicating that the enzyme had attached to the capillary endothelium. Hepatic lipase deficient mice injected with HL rAdV had a peak (day 4- 5) reduction in TC (-23%, $p<0.001$), PL (-30%, $p<0.002$) and FC (-35%, $p<0.05$). Plasma analysis on FPLC showed dramatic decreases in HDL-C and PL as well as the formation of LDL-sized cholesterol and phospholipid enriched particles in rAdV treated mice. Thus, gene transfer using recombinant adenovirus resulted in successful replacement of HL lipase in HL deficient mice as well as normalization of the lipoprotein phenotype.

Annual Report of the Molecular Hematology Branch
National Heart, Lung, and Blood Institute
October 1, 1993 - September 30, 1994

This report covers the activities of the Section on RNA and Protein Biosynthesis and the Section on Molecular Genetics. Increased emphasis has been directed towards the development of basic knowledge and technology required for the successful implementation of therapeutic concepts to human gene therapy.

SECTION ON RNA AND PROTEIN BIOSYNTHESIS

This section studies the mechanisms by which expression of the alpha and beta subunits of the initiation factor eIF-2 is regulated. A second major area examines the molecular basis of adeno-associated virus (AAV) integration; in addition, a detailed biochemical examination of the non-structural Rep proteins' functions is being made.

During the past year this section has achieved the following major goals:

1. The complete eIF-2β gene has been cloned. A consensus α-PAL binding site is now identified in both the α and β subunits of eIF-2 and may play a role in their coordinate expression.

2. The cDNA of α-PAL has been cloned and characterized as encoding a 503-amino acid polypeptide which binds to the consensus DNA recognition sequence.

3. Using an EMSA technique involving selection of binding sequences from a random oligonucleotide pool, the consensus DNA-recognition sequence for α-PAL has been determined to be T/C G/C GCAT/C GCGCA.

4. α-PAL is identified as a member of the basic leucine zipper class of transcription factors which bind to DNA as a homodimer.

5. Forty genes containing the α-PAL binding motif in their promoter region can be classified as growth responsive genes.

6. Guanine nucleotide exchange activity of eIF-2B increases 10-fold within the first 15 minutes of T-cell activation.

7. The immunosuppressants FK506 and rapamycin inhibit the translation of specific proteins during T-cell activation. Jurkat cells arrested in G_1 with anisomycin show an almost total inhibition of translation following rapamycin treatment.

8. Four polyadenylation sites have been identified within the 3'-UTR of the 4.2 kb mRNA of eIF-2α. Differential utilization of these sites is responsible for the 1.6 kb and 4.2 kb mRNA species found in different tissues. Differential utilization appears to be correlated with a high degree of secondary structure.

9. The AAV nonstructural protein Rep68 binds to a $(GCTC)_4$ repeat motif in the linear form of the ITR with equal affinity as to the hairpin ITR. The Kd determined for binding to both forms of the ITR is 8×10^{-10}M.

10. Although Rep68 binds with equal affinity to the linear and hairpin ITR structures, terminal resolution site activity is 100-fold less efficient in the linear form of the ITR.

11. Recombinant Rep68 can allow replication and resolution of recombinant AAV sequences in in vitro assays utilizing uninfected HeLa whole cell extracts.

12. A new terminal resolution assay has been developed which measures the covalent linkage of Rep68 to the 5' base in the terminal resolution site.

13. In vitro integration of 3'-labeled ITR into the P1 sequence of the chromosome 19 AAV integration site has been achieved.

14. In vitro AAV replication of a plasmid is shown to require Rep68 and the P1 sequence containing the GCTC repeat motif.

15. Rep68 is shown to promote integration of ITR containing sequences by binding to two almost identical binding sites present in the P1 and ITR sequences.

SECTION ON MOLECULAR GENETICS

This section investigates the application of gene transfer techniques to the understanding of cardiovascular biology and the treatment of cardiovascular disease.

During the past year this section has achieved the following major accomplishments and discoveries:

1. Transfer of plasminogen activator genes into primate endothelial cells followed by seeding of cells onto thrombogenic vascular graft segments increased t-PA and urokinase secretion 10-fold.

2. In an in vivo baboon thrombosis model, platelet and fibrin deposition were significantly decreased by seeding of graft segments with genetically modified endothelial cells expressing t-PA or urokinase.

3. We confirmed in vivo retention of genetically modified endothelial cells seeded onto prosthetic vascular grafts 2 hours following placement of seeded grafts in the in vivo arterial circulation of sheep.

4. We constructed retroviral and adenoviral vectors that express biologically active hirudin. Human endothelial cells transduced with the retroviral vector secrete hirudin at stable rates over at least 4 weeks in vitro. Human endothelial cells transduced with the adenoviral vector secrete hirudin at very high levels, however, the use of adenoviral vectors is limited by in vitro cellular toxicity.

5. We optimized adenovirus-mediated gene transfer into injured rat carotid arteries. The efficiency of gene transfer in this system was limited by acute local toxicity characterized by smooth muscle cell death and inflammatory infiltrates. This acute toxicity currently places an upper limit on the levels of gene transfer achievable with adenoviral vectors in vivo.

6. A heterologous signal peptide was identified that confers high level secretion and proper N-terminal processing on hirudin protein expressed from viral vectors in human endothelial cells. Using an adenoviral vector containing this peptide sequence, we achieved in vivo expression of hirudin from endothelial cells in the rat arterial wall. As hirudin is an antithrombotic agent with proven therapeutic value in both humans and animal models, we anticipate that local expression of hirudin by gene transfer will be a useful approach to the prevention of thrombosis.

7. We developed and optimized a unique animal model system of vascular endothelial cell-specific gene transfer. This in vivo gene transfer system will be useful both in studying mechanisms of endothelial cell gene regulation in vivo and in defining the biological roles of endothelial cell-specific gene products.

8. Incorporation of a regulatable promoter sequence into an adenoviral vector resulted in appropriate physiological regulation of the expression of a reporter gene in cultured endothelial cells. Use of this promoter sequence may allow regulated transgene expression in endothelial cells in vivo.

9. Significant levels of gene transfer were achieved following delivery of adenoviral vectors into the pulmonary arteries of rats. This observation is potentially of major importance for the development of genetic therapies for pulmonary hypertension, an essentially untreatable human disease.

Annual Report of the Pathology Branch
Division of Intramural Research
National Heart, Lung, and Blood Institute
October 1, 1993 to September 30, 1994

The Pathology Branch maintains a broad range of research interests in Cardiovascular and Pulmonary Pathology. During the past year, research studies were completed on different aspects of 1) the dilated cardiomyopathy associated with chronic Chagas' disease; 2) prevention of the cardiomyopathy associated with the administration of doxorubicin for the treatment of metastatic neoplasms; 3) the cardiotoxicity of cytokines; 4) prosthetic heart valves; and 5) interstitial lung diseases.

Chronic Chagasic Cardiomyopathy

Because of the public health importance of Chagas' disease, which is considered to affect approximately 20 million individuals in the American continent, we have developed a research interest in the pathology and pathogenesis of this disorder. We have previously described the histologic and ultrastructural changes that can be observed in myocardial biopsy specimens taken from patients with chronic chagasic cardiomyopathy. These studies have shown that myocardial damage, inflammation and fibrosis develop gradually as the result of immunologic mechanisms that do not seem to involve the continuing presence of the parasites (Trypanosoma cruzi amastigotes) in the cardiac myocytes. We have also shown that marked thickening of the basement membranes of cardiac myocytes, endothelial cells and vascular smooth muscle cells constitutes a characteristic feature of the disease. This year, we completed an immunohistochemical study designed to evaluate the presence of laminin, a connective tissue glycoprotein normally present in basement membranes, in these thickened basement membranes. These studies, performed on endomyocardial biopsy specimens from ten patients with well documented chronic Chagasic cardiomyopathy, utilized peroxidase-and gold-labelled antibodies. The results obtained showed that the myocardium of patients with chronic chagasic cardiomyopathy contains very large amounts of laminin, which is present not only throughout the thickened basement membranes but also in amorphous deposits in the myocardial interstitium. The finding of increased amounts of laminin is of special importance because other studies have shown that sera from patients with chronic chagasic cardiomyopathy often have high titers of circulating antibodies against laminin. The thickening of basement membranes obseved in this study may develop as a consequence of: a) an immunologic reaction which is triggered by the presence of a laminin-like molecule on the surfaces of T. cruzi amastigotes and trypomastigotes; b) an immunologic response to direct injury of basement membranes causing some of their components to become antigenic; c) myocardial fibrosis, with synthesis of new connective tissue components, and d) a combination of the preceding factors.

In another study completed this year, an attempt was made to correlate the extent to which antilaminin antibodies are present in the sera of chagasic patients with various clinical and laboratory parameters pertinent to their heart disease. The highest titers of antilaminin antibodies were found in the patients with the most

severe forms of the disease; however, no correlation could be established between the titers of these antibodies and the results of other laboratory studies.

The studies just reviewed point to the need for an experimental animal model in which details of these pathologic processes can be evaluated in the context of the natural history of Chagas' disease. In the past, it has proven extremely difficult to duplicate all the features of human chronic Chagasic cardiomyopathy in experimental animals. A notable breakthrough in this respect has been achieved by the group headed by Dr. Zilton Andrade in Brazil. These investigators have succeeded in developing an excellent animal model in dogs that are inoculated with the 12 SF strain of T. cruzi. We have developed a plan of collaboration with Dr. Andrade's group to study the histologic, immunohistochemical and ultrastructural features of the heart in this animal model. The first of these collaborative studies was completed this year and consisted of an evaluation of the changes observed during the acute phase of Chagas' disease (acute myocarditis; up to one month after inoculation). These studies have clarified two aspects of the disorder: 1) that much of the myocardial damage observed in the acute phase is due to the cytotoxic effects of lymphocytes and macrophages that recognize myocytes as targets and then induce their damage and/or destruction, and 2) that the microcirculation is also effected by the cytotoxicity of inflammatory cells. Studies of subsequent phases of Chagasic cardiomyopathy in dogs are currently in progress, together with a reevaluation of the role of immune effector cells in the human counterpart of this disease.

Studies on Doxorubicin-induced Cardiomyopathy

The clinical use of new, powerful chemotherapeutic agents is often associated with clinically significant cardiac toxicity, and this unit has made intensive studies of the pathogenesis and prevention of these adverse effects. This unit has maintained a long-term interest in the cardiomyopathy produced by doxorubicin, an anthracycline compound that is highly effective in the chemotherapy of a variety of malignant tumors both in children and in adults. We have previously developed experimental animal models, especially in spontaneously hypertensive rats (SHR), for the study of this cardiotoxicity. These studies have demonstrated that a major factor in the pathogenesis of this cardiotoxicity involves the formation of a doxorubicin-iron complex that catalyzes the generation of oxygen free radicals. The cytotoxic effects of these radicals result in cumulative, dose-dependent damage to the cardiac myocytes. The reversibility of this damage remains uncertain. A semiquantitative assessment of the damage to cardiac myocytes can be made on the basis of morphologic observation of the heart to determine the percentages of myocytes showing myofibrillar loss and dilitation of the sarcoplasmic reticulum. These alterations constitute a characteristic histologic "marker" for doxorubicin cardiotoxicity. The prevention of this damage is an important issue in cancer chemotherapy, and we have previously provided evidence demonstrating that doxorubicin-induced cardiac toxicity can be minimized by the concomitant administration of ICRF-187 (formula [(D)1,2-bis(3,5-dioxopiperazinyl-1-yl)propane]). This drug appears to function as an iron chelator, thus resulting in a

decrease in the iron-catalyzed formation of free radicals that induce damage. We have previously shown that ICRF-187 can be used successfully in human patients undergoing cancer chemotherapy. However, a number of important questions still need to be addressed concerning the best approach to the prevention of doxorubicin cardiotoxicity. Several studies pertinent to the cardioprotective action of ICRF-187 were conducted this year. The first of these involved a comparison of the cardioprotection provided by ICRF-187 and ICRF-186, its L-enantiomer. We have shown that ICRF-186 is highly effective in the mitigation of the acute toxicity of doxorubicin and daunorubicin, as demonstrated in a comparative study of the cardioprotective activity of a number of chemical analogues of ICRF-187. Based on these preliminary findings, we investigated the degree of cardiac protective activity of ICRF-186 in the SHR model of chronic toxicity of doxorubicin. This study showed that ICRF-186 is slightly less cardioprotective than ICRF-187 when the two drugs are given under identical circumstances. Thus, the use of ICRF-186 does not offer any significant chemotherapeutic damage over that of its D-enantiomer. In another study, also conducted in SHR, a comparison was made of the protective activity of ICRF-187 and desferrioxamine (the only other iron chelator currently approved for clinical use in patients) against the cardiac toxicity of doxorubicin. The study showed that desferrioxamine is considerably less effective than ICRF-187 in reducing this toxicity. The effectiveness of desferrioxamine was improved by using a conjugate of the drug with hydroxyethyl starch. The resulting compound has a much longer plasma half-life than does desferrioxamine mesylate, the standard form of the drug. Analysis of the data suggested that, although desferrioxamine is a very highly effective chelator of iron, it does not penetrate readily into cells. Therefore the intracellular pools of iron that must be chelated to decrease the cardiotoxicity of doxorubicin are not readily accessible to desferrioxamine.

Beagle dogs were employed, using a model system that we have previously developed, to assess the effects of varying the time interval between the administration of ICRF-187 and that of doxorubicin. The results of the study showed that the effectiveness of ICRF-187 is maximal when the drug is administered either one hour before, or concurrently with, doxorubicin. Decreased effectiveness of ICRF-187 was demonstrated when this drug was given after doxorubicin. These observations are of importance in the scheduling of the administration of these two drugs in clinical practice.

Cardiotoxicity of Interleukin-2

During the past few years, a number of studies have shown that the administration of interleukin-2 constitutes an effective mode of therapy in patients with diverse types of disseminated neoplasms. Nevertheless, the use of this agent is now known to be associated with a significant incidence of cardiac and pulmonary complications, mainly the vascular leak syndrome. This syndrome is characterized by myocarditis, myocardial necrosis, congestive heart failure, hypotension, and pulmonary and peripheral edema. Evidence derived from studies in humans and in experimental animals has been interpreted as indicating that

damage to cardiac myocytes and vascular structures results from the induction of lymphokine activated killer cells (LAK) cells. Such cells establish contacts with, and exert cytotoxic effects on, the endothelial cells and subsequently migrate into the interstitium, where they also induce damage to cardiac myocytes. Preliminary studies showed that the severity of the vascular leak syndrome induced by the administration of interleukin-2 can be modified by the coadministration of other cytokines. Therefore detailed studies were undertaken to evaluate the effects of these therapeutic manipulations on the structure of the heart, lungs, liver and kidney of mice treated with interleukin-2 in a manner similar to that used clinically. These studies included the evaluation of the effect of interleukin-α and interferon-α singly and in combination. Interleukin-2 induced a vascular leak syndrome of a moderate severity, with infiltration of lymphoid cells, moderate endothelial damage, mild hepatic parenchymal damage and minimal myocardial alterations. Interferon-α produced infiltration mainly of monocytes/macrophages in liver and heart; endothelial cell damage was absent in lung and heart, and minimal in liver. Interleukin-1α caused an increased number of neutrophils in liver and lung; vascular leak syndrome and parenchymal cell and endothelial damage were not found. The vascular leak syndrome and the cellular damage caused by the combination of interleukin-2 and interferon-α were much more severe than those produced by interleukin-2 alone. In animals treated with interleukin-2, interferon-α and interleukin-1α, the vascular leak syndrome was minimal and parenchymal and endothelial cell damage were less severe than after interleukin-2 alone or interleukin-2 plus interferon-α. Taken together, these observations show that interleukin-1α reduces the severity of the ultrastructural changes produced by interleukin-2 and interferon-α. This reduction may be clinically useful in the treatment of neoplasms.

During the past year, we have developed new methods for the simultaneous identification of three different types of immune effector cells in cardiac and pulmonary tissues in rats. At the present time, our laboratory is capable of performing immunohistochemical staining reactions for lymphokine activated killer cells, interstitial dendritic cells and macrophages in the same tissue section. This technology is now beginning to be used for the quantitative estimation of different types of cell populations that infiltrate the heart muscle in the lungs after the administration of cytokines. In addition, similar studies are being undertaken using animal models of myocarditis. These techniques involve the combined use of antibodies variously labelled with fluorescent compounds, alkaline phosphatase and perioxadase. The application of automated methods of cell counting, using color television microscopy, to preparations stained by these multiple labelling technics is beginning to provide highly useful information that otherwise cannot be obtained.

Prosthetic Heart Valves

For the past twenty years, this unit has been involved in the evaluation of many types of prosthetic heart valves. We have made extensive studies of the structure of unimplanted heart valves, particularly of valves made of suitably processed animal tissues (such as glutaraldehyde-treated porcine aortic valves and

bovine parietal pericardial valves), and we have established a number of colorations between the structural and functional characteristics of these valves. This year, an extensive review was made to provide a comprehensive evaluation of currently available models of prosthetic heart valves with flexible leaflets. The study was based on the experience of this unit, including examination of several hundred artificial heart valves explanted from patients and from experimental animals. The main pathologic findings in these valves consisted of calcification, primary leaflet failure, fibrous sheathing and thrombus formation, all of which contributed to prosthetic valve failure. Emphasis was placed on the importance of the selection of biomaterials, based on their chemical, physical and mechanical properties and on knowledge of the alterations induced in these properties by preimplantation processing. This review provides guidance for the continuing development of the next generation of prosthetic heart valves, utilizing the experience gained from previous preclinical and clinical studies.

A new method was developed for the simultaneous quantitation of porcine aortic valve collagen crimp length and the assessment of biomechanical properties. Previous studies from this unit have shown that the extent of crimping (waviness) of the collagen fibrils in leaflet tissue is an important determinant of the biomechanical properties of tissue heart valves. Nevertheless, the quantitative assessment of collagen crimping in heart valves has presented very difficult technical problems. We have solved some of these problems by the application of techniques of polarized light microscopy to the study of these valves in preparations that include either entire leaflets or very large portions of leaflet tissue. Our new method utilizes the simultaneous real-time video recording of collagen crimp morphology and acquisition of crimp length data through the combination of polarized light microscopy and morphometry. We felt that the development of this method was warranted due to the fundamental role played by collagen in the mechanical performance of tissue valves. The development of this method involved the design and fabrication of a uniaxial microtensile stage, suitable for mounting on a standard microscope stage. The validation of our test method was accomplished by a comparison of untreated and glutaraldehyde-treated porcine aortic valve leaflet tissue, since the biomechanical and morphologic characteristics of the native and fixed porcine aortic valve have been extensively studied. This method enables the collection of morphologic and biomechanical data from a single tissue specimen, eliminating the need for independent studies of multiple specimens. Furthermore, this method obviates the need for making assumptions, which may be difficult to verify, concerning the homogeneity of different test specimens with respect to their morphology and corresponding mechanical response to different experimental conditions.

Interstitial Lung Disorders

The interstitial lung disorders, especially idiopathic pulmonary fibrosis, continue to be of great interest to the Pathology Branch. In previous years, we have provided extensive, detailed data on the histologic and ultrastructural changes that occur in the lungs of patients with various pulmonary interstitial disorders. In

addition, we have studied experimental animal models of these conditions, mainly for the purpose of evaluating some of the pathogenetic mechanisms involved in the fibrosis. These studies have shown that the initial stages of pulmonary fibrosis are characterized by intraalveolar (intraluminal) fibrosis, in which interstitial myofibroblasts migrate through defects in the epithelial basement membrane into the alveolar lumina, where they synthesize collagen and other connective tissue proteins. These intraalveolar masses of myofibroblasts and newly synthesized fibrous tissue become incorporated into the interstitial space when they become lined by regenerating epithelial cells. This appears to be the basic mechanism by which pulmonary structural remodeling occurs in fibrotic lung disorders. Having studied the temporal sequence of these ultrastructural changes, we are now turning to an assessment of the immunohistochemical alterations that accompany these processes. The initial phase of these studies consisted of an evaluation of the significance of early intraalveolar fibrosis and immunohistochemically demonstrable fibronectin receptors in lungs of patients with idiopathic pulmonary fibrosis. To study the pulmonary structural remodeling in idiopathic pulmonary fibrosis, ultrastructural, immunohistochemical and light microscopic morphometric observations were made on 11 pulmonary biopsies from patients with idiopathic pulmonary fibrosis. The morphometric study was made using sequentially cut tissue sections stained for keratin-Alcian blue PAS, fibronectin and type IV collagen-Alcian blue PAS. Most of the early fibrotic lesions, which were Alcian blue- and fibronectin-positive, were intraalveolar in location. A strong reaction for integrin $\alpha 5\beta 1$ and vinculin was found in epithelial cells and mesenchymal cells in areas of intraalveolar fibrosis. These findings show that these cells are active in adhesion to fibronectin in areas of early intraalveolar fibrosis. Some of the epithelial cells, including cytoplasmic hyaline-laden cells, showed evidence of inadequate adhesion to the extracellular matrix, and this may constitute one of the mechanisms of progression of fibrosis in idiopathic pulmonary fibrosis. The results of this study emphasize that intraalveolar fibrosis is a necessary prerequisite for the development of interstitial fibrosis with pulmonary remodeling, and that $\alpha 5\beta 1$ integrin is expressed strongly by epithelial and mesenchymal cells in areas of intraalveolar fibrosis. These observations also suggest that inadequate adhesion of new epithelial cells to the underlined extracellular matrix may contribute significantly to the progression of intraalveolar fibrosis and alveolar luminal obliteration. Details of these events continues to be the subject of critical analysis.

Annual Report of the Pulmonary Branch
National Heart, Lung, and Blood Institute
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In the past year, the Pulmonary Branch basic and clinical research program was focused on two areas: I. Gene therapy, including in vivo gene transfer strategies for the lung and other organs; and II. The roles of nitric oxide in acute and chronic lung injury. The gene therapy protocol was the continuation of studies initiated by the former Chief, Dr. Ronald G. Crystal. The studies on the roles of nitric oxide were initiated during this fiscal year.

I. Gene Therapy

CF is an autosomal recessive disorder resulting from mutations of the cystic fibrosis transmembrane regulator (CFTR) gene, a 27 exon gene occupying 250 kb of chromosome 7. All organs with exocrine glands are affected, but the major clinical manifestations are in the lung, with impacted mucus, chronic infection, inflammation, and airway and parenchymal lung derangements. Studies in the past year regarding gene therapy for cystic fibrosis have focused on gene therapy for the potential treatment of the respiratory manifestations of the disease.

A strategy for somatic gene therapy of CF is direct transfer of the normal CFTR gene to CF airway epithelial cells using a replication deficient recombinant adenovirus (Ad) vector. Given that in vitro transfer of normal CFTR cDNA corrects the defect and that the recombinant adenovirus (Ad), AdCFTR, transfers the human CFTR cDNA in vivo to the airway epithelium of experimental animals, CF may be amenable to Ad-mediated gene therapy.

(A). Following approval by the appropriate regulatory groups, the first human gene therapy trial for CF was begun on April 17, 1993. AdCFTR was administered to the nasal and bronchial epithelium of four CF individuals (1A,1B,2A,3A). The doses of AdCFTR (plaque forming units cpfu/ml) for each individual were: 1A. nasal, $2x10^5$; bronchial, $2x10^7$; 1B. nasal, $2x10^5$; bronchial, $2x10^7$; 2A. nasal, $2x10^7$; bronchial, $2x10^9$; 3A. nasal, $2x10^7$; bronchial, $2x10^6$. The data demonstrate that an adenovirus vector transferred the CFTR cDNA to the respiratory epithelium of individuals with CF in vivo. After administration of AdCFTR to the respiratory epithelium, AdCFTR DNA was detectable in bronchial epithelium of individual 1B and nasal epithelium of individual 3A, and AdCFTR-directed mRNA was detectable in nasal epithelium of individual 1A. Importantly, after administration of AdCFTR to respiratory epithelium, CFTR protein was detectable with the anti-CFTR antibody in nasal epithelium of individual 1A and bronchial epithelium of individual 3A. With doses of $2x10^6$ to $2x10^9$ pfu administered to the bronchial epithelium, there was no evidence of recombination or complementation of the vector to produce a replication-com-

petent adenovirus, shedding of the vector, or rise in titers of serum anti-adenovirus neutralizing antibodies. At the highest dose ($2x10^9$ pfu), there was a transient systemic and pulmonary syndrome [including headache, fatigue, fever, tachycardia, dyspnea, hypoxemia, pulmonary infiltrates, and hypotension (not requiring vasopressor therapy)] likely secondary to local inflammation initiated by the vector in the lower respiratory tract, mediated in part by interleukin-6. Despite this, 6 to 12 month follow-up of all four patients demonstrated no long-term adverse effect, with lung function tests similar to pre-therapy values.

(B). In studies using AdCFTR to evaluate CFTR functions other than that of a cAMP-regulated Cl^- channel, cell staining of dihydrorhodamine 6G (dR6G), a known substrate for MDR1, was increased in cells overexpressing CFTR. Importantly, this staining was opposite to that observed in cells overexpressing MDR1, i.e., MDR1 cells stained to a lesser extent with dR6G. In addition, staining in CFTR-expressing cells was not influenced by known inhibitors of MDR1 function. Thus, CFTR, in addition to acting as a chloride channel, is capable of influencing the level of dR6G staining. Also, CFTR function (monitored in whole cells using the anion sensitive dye SPQ) was increased with decreasing intracellular glutathione and decreased slightly with increasing intracellular glutathione.

(C). In the study of gene therapy for acute lung injury, AdSP vectors were capable of expressing the surfactant cDNA in Cos1 and A549 cells in vitro (AdSP-A, AdSP-B and AdSP-C), and the lungs of Sprague-Dawley rat in vivo (AdSP-A and AdSP-B).

II. Role of Nitric Oxide in Acute and Chronic Lung Disease

Nitric oxide (NO), a molecule with diverse functions (e.g., macrophage activation, vasodilation, neurotransmission), is a small, uncharged, highly-reactive free radical (half life of 2 to 30 sec), synthesized from L-arginine by nitric oxide synthase (NOS). Three isoforms of NOS have been identified based on structure and chromosomal localization. Two subtypes are constitutive, calcium-dependent enzymes found in endothelium and neurons, respectively. The other subtype is an inducible enzyme (iNOS) which, following appropriate stimuli (e.g., induction with cytokines), is increased in many mammalian cells (e.g., macrophages, neutrophils, hepatocytes, cardiac myocytes, and endothelial cells).

The lung contains various cells known to produce NO, which are critical for host defense and injury and for vascular and neuronal regulation. It is the first line of defense against exogenous NO from air pollutants and cigarette smoke. Finally, the lung is an oxygen-rich environment, allowing for the reaction of NO with oxygen-derived molecules, resulting in highly reactive compounds. Since NO is hydrophobic, it can act as both an intracellular and intercellular messenger.

To help elucidate the role of NO in signalling and in the pathogenesis of lung diseases, an animal model for NO-induced lung damage was developed as an adjunct to human studies to evaluate NO production in disease models; and to assess lung damage associated with NO synthesis. NO production was evaluated in male Sprague-Dawley rats receiving lipopolysaccharide (LPS) from E. coli (serotype 0111:B4). iNOS RNA was detected only in RNA extracted from animals injected with endotoxin.

Supernatant from alveolar macrophages, isolated from lavage fluid and cultured for 24 hours, was evaluated for the presence of nitrite, an end-product of NO production. Nitrite level was higher (compared to control animals) in the supernatant from alveolar macrophages from animals injected with endotoxin. Furthermore, nitrite levels were higher in lavage fluid from animals treated with endotoxin compared to control animals. Morphologic evaluation of lung tissues revealed that animals injected with endotoxin had vascular congestion, neutrophil accumulation in parenchymal tissues, and neutrophil margination in the pulmonary blood vessels.

ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
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NK-2 Homeobox Gene. The NK-2 homeobox gene of Drosophila is the earliest predominantly neural gene regulator that has been found thus far that is expressed in the ventrolateral neurogenic anlage, which gives rise to part of the CNS of the embryo. Genes for proteins that regulate NK-2 gene expression were identified by determining the patterns of NK-2 mRNA in wild type and mutant lines of Drosophila as a function of developmental age. The NK-2 gene was shown to be activated initially by dorsal in the ventral half of the embryo at the syncytial blastoderm stage. However, in wild type embryos the NK-2 gene is not expressed in the mesodermal anlage due to repression by snail, in the mesectodermal anlage due to repression by single-minded (sim), or in the lateral neuroectodermal or dorsal epidermal anlagen due to repression by an unknown gene regulator mediated by decapentaplegic (dpp). Both dorsal and twist are required to activate the NK-2 gene in the hindgut and posterior midgut primordia. Proneural gene mutants such as daughterless or deletion of the Achaete-Scute complex of genes, which encode basic helix-loop-helix (HLH) DNA binding proteins, affect the expression of the NK-2 gene only slightly. However, the number of cells that synthesize NK-2 mRNA was reduced markedly in a ventral nervous system defective mutant. NK-2 gene expression responds directly to the ventral-dorsal concentration gradient of dorsal and probably indirectly to anterior-posterior gradients of gene regulators. Therefore, NK-2 gene expression is restricted to nuclei in the ventral half of the neurogenic anlage by repression. The ventral and dorsal borders of the NK-2 positive stripe of nuclei are formed independently by different species of repressor. The position of the NK-2 positive stripe of nuclei on the ventral-dorsal axis of the embryo and the width of the stripe are not fixed but instead depend upon the combined effects of proteins that activate and repress the NK-2 gene. The results show that regulation of NK-2 gene expression is hierarchically organized based on the positions of nuclei on the ventral-dorsal and later on the anterior-posterior axes of the embryo. Many of the neuroectodermal cells that express the NK-2 gene develop into medial neuroblasts and posterior compartment neuroblasts. The NK-2 gene also is expressed by some ganglion mother cells and neurons, presumably the progeny of NK-2 positive neuroblasts. Some NK-2 positive neurons contribute to the commissures and longitudinal connections of the ventral nerve cord. NK-2 mRNA is present in the nervous system of embryos, but little or none was found in larvae or pupae, which suggests that NK-2 homeodomain protein plays a role in the assembly rather than the activity of the nervous system. Deletion of the NK-2 gene and some neighboring genes results in embryos with grossly defective ventral nerve cords that contain few neurons, which suggests that NK-2 may be a proneural gene required for the development of medial neuroectodermal cells and neuroblasts. Currently we are trying to obtain specific mutations of the NK-2 gene.

Initially all nuclei in the ventrolateral neurogenic anlage are committed to the neuroblast pathway of development. However, only about 50% of the neuroectodermal cells segregate as neuro- blasts; most of the remaining neuroectodermal cells switch from the neuroectodermal to the epidermoblast pathway of development due to contact between the neuroectodermal cell and a neuroblast, a process termed lateral inhibition. Genes required for lateral inhibition include Delta and the Enhancer of split (E(spl)) complex which contains a cluster of genes that encode similar HLH proteins that are required for epidermoblast development. Mutation of the Delta gene or deletion of the E(spl) gene complex resulted in the ectopic expression of the NK-2 gene in mesectodermal cells, in addition to expression in neuroectodermal cells, and in the production of extra thoracic neuroblasts that express the NK-2 gene, which suggests that E(spl) HLH proteins repress the NK-2 gene. Delta probably represses NK-2 indirectly by signalling activation of E(spl) genes. These results suggest that lateral inhibition results in repression of the NK-2 gene.

Randomly ordered oligodeoxynucleotides that bind to the NK-2 homeodomain were purified, amplified, cloned, and sequenced. The consensus nucleotide sequence for NK-2 homeodomain binding was shown to be TNAAGTGG and the KD, 2 x 10(-10) M. 13C and 15N labelled NK-2 homeodomain proteins were synthesized in E. coli, purified, and used to determine the secondary structure of the NK-2 homeodomain by NMR. Three α-helical segments including a helix-turn-helix motif were found. The stability of NK-2 homeodomain secondary structure increased markedly upon binding to DNA and the length of α-helix 3 increased from 11 to 19 amino acid residues. Methylation of N7 of guanine and N3 of adenine by dimethylsulfate interferes with contacts between protein and guanine or adenine residues in the major or minor grooves of DNA, respectively. Methylation of A-4, A-5, and G-6 of (+) 5-TTGAAGTGGA-3 and A-1, A-2, and A-7 of the complimentary strand, (-) 3-AACTTCACCT-5, interfered with binding of the NK-2 homeodomain to DNA. In addition, contacts between the NK-2 homeodomain and the DNA phosphodiester backbone were identified by ethylation interference analysis.

Three overlapping Drosophila genomic DNA clones were obtained containing 2.2, 8, or 14 kb of DNA from the 5-upstream region of the NK-2 gene. Nucleotide sequence analysis of 2.2 kb of the 5-flanking region of the NK-2 gene revealed many putative binding sites for proteins that regulate gene expression including dorsal, NK-2, and snail. Two DNA fragments (2.2 and 8.4 kb) from the 5-upstream region of the NK-2 gene were subcloned in a vector that contains a chloramphenicol acetyltransferase (CAT) reporter gene. Schneider S2 cells were cotransfected with an NK-2 CAT vector construct and a vector containing constitutively expressed dorsal cDNA. Dorsal protein strongly activated the expression of the CAT reporter gene in plasmids that contained 2.2 or 8.4 kb of DNA from the upstream region of the NK-2 gene.

Drosophila HMG-D Gene. A transgenic line of Drosophila was obtained that contains a P-element with a β-galactosidase reporter gene inserted 1.3 kd upstream

of the HMG-D gene, which encodes a chromosomal protein. The HMG-D gene is ubiquitously expressed during early embryonic development but later in development is restricted to the nervous system. HMG-D protein was synthesized in E. coli, purified, and was shown to have a higher affinity for cruciform DNA than for double-stranded DNA. Thus HMG-D protein recognizes DNA conformation preferentially, rather than nucleotide sequence. The homozygous P-element insertion is lethal and results in embryos with striking defects in the ventral nerve cord.

Zinc Finger Gene 367. Transgenic Drosophila line 367 was shown to contain a P-element inserted in the flanking region of novel zinc finger gene 367. The expression of zinc finger gene 367 is restricted to a subset of cells in the CNS and anterior sensory organs. The P-element insertion is a recessive lethal mutation; embryos with homozygous P-element insertions exhibit massive defects of the ventral nerve cord.

Novel Mouse Homeobox Genes. Six novel mouse homeobox genes, OG-2, OG-9, OG-12, OG-22, OG-35, and NKx-1.2 were cloned and characterized. Sequence analysis showed that OG-35 encodes a protein with a homeodomain that is 88% identical to the C. elegans unc-4 homeodomain, which determines part of the synaptic circuit of a class of motorneurons. The abundance of OG-35 RNA was maximal in 10-12 day mouse embryos. OG-35 mRNA was detected by Northern analysis of RNA from adult mouse brain, spleen, lung, liver, and kidney and from NG108-15 neuroblastoma-glioma hybrid cells. The major species of OG-35 mRNA is approximately 1 kb in length, a longer species of OG-35 RNA also was found in brain.

The OG-12 homeodomain is not closely related to any previously reported homeodomain but exhibits some homology to the homeodomains of pax-3 (72% homology) and OG-35, unc-4, smox-3, and S8 (70% homology). Multiple species of OG-12 poly A+ RNA were found in 10-16 day mouse embryos, which suggests alternative splicing. One major species of OG-12 mRNA, 1.8 kb in length, was detected in adult mouse skeletal muscle. Two species of OG-12 cDNA, which correspond to alternatively spliced species of OG-12 mRNA were cloned from a mouse embryo cDNA library and were sequenced. In addition 3,455 base pairs of OG-12 mouse genomic DNA were sequenced.

OG-22 is similar to the recently described rat cartilage-1 homeobox cDNA. We find two species of OG-22 cDNA which correspond to two alternatively spliced species of OG-22 mRNA.

The OG-2 homeodomain is not closely related to any previously reported homeodomain but exhibits some homology (61%) to OG-12 and the Drosophila aristaless homeodomains. OG-2 mRNA is expressed in 12-16 day mouse embryos. Two major species of OG-2 mRNA were detected in adult mouse skeletal muscle and trace expression of OG-2 mRNA was found in cardiac muscle.

OG-9 genomic DNA was cloned and 1272 bp was sequenced. One species of OG-9 mRNA approximately 1 kb in length was found in adult mouse skeletal muscle; but OG-9 mRNA was not detected in many other tissues tested. The OG-9 homeobox gene therefore encodes a striated muscle specific homeodomain protein, which is not closely related to any known homeodomain.

A novel mouse homeobox gene was cloned (NKx-1.2) that encodes a homeodomain that is closely related to the Drosophila NK-1 and chicken Chox-3 homeodomains. Eight kb of NKx-1.2 genomic DNA were sequenced. The deduced amino acid sequence of the NKx-1.2 homeodomain differs from the Drosophila NK-1 and chicken Chox-3 homeodomains by only 3 and 1 amino acid residues (95 and 98% homology, respectively). NKx-1.2 poly-A+ RNA was detected in 10-18 day mouse embryos. Northern analysis of poly-A+ RNA from adult mouse tissues revealed 1 major band of NKx-1.2 RNA in RNA from brain and trace bands in RNA from testes and spleen In situ hybridization revealed NKx-1.2 RNA in the mesencephalon, myelencephalon, spinal cord, vertebrae, and ribs.

Hoxd 3 and Hoxa 3. Mouse Hoxd 3 genomic DNA and cDNA and Hoxa 3 cDNA were cloned and sequenced. Only the sequences of the homeobox regions of Hoxd 3 and Hoxa 3 have been reported previously. The deduced complete amino acid sequence of Hoxd 3 protein consists of 417 amino acid residues. Hoxd-3 and Hoxa-3 proteins contain tandem putative binding sites for the src homology 3 domain; however, we do not yet know whether Hoxd 3 or Hoxa 3 homeodomain proteins respond to extracellular signals mediated by the src protein kinase. Northern analysis showed that 2 species of Hoxd 3 mRNA, 4.3 and 3 kd in length, are expressed in 8-11 day old mouse embryos. Hoxd 3 mRNA is expressed in mouse embryo mylencephalon, spinal cord, thyroid, kidney, esophagus, stomach, and intestine; however in the adult, Hoxd 3 mRNA was found only in the kidney. The nucleotide sequence of the coding region of Hoxa 3 was extended considerably and numerous errors were found in the amino acid sequence of the Hoxa 3 homeodomain that had been reported by others.

POU-Domain Genes. Previously we cloned and sequenced four mouse class III POU domain genes, Brain-1, Brain-2. Brain-4, and SCIP. The distribution of mRNA for each of the 4 POU-domain genes was defined as a function of developmental age by in situ hybridization. Brain-2 expression was shown to be restricted to the nervous system. Brain-1, Brain-4 and SCIP genes were shown to be expressed predominantly in the nervous system but also were found in other organs. Each of the 4 genes exhibit different spatial and temporal patterns of expression, which overlap in various subsets of cells at almost all stages of neural development. Early in embryonic development the expression of each gene changes dyamically with region-specific differentiation of neuroepitheleal cells, migration of neurons and glia, and terminal differentiation of post-migratory cells. At later stages of development, Brain-2, Brain-4, and SCIP mRNA are restricted to distinct cell groups in various regions of the nervous system. In contrast, Brain-1 expression expands gradually throughout the entire nervous system and is seen in almost all neurons in the adult CNS.

Postsynaptic Development of the Neuromuscular Junction.
We carry on basic research into the organization of the excitable membranes and myofibrils of striated muscle and the cellular and molecular mechanisms involved in their differentiation. We focus on two membrane systems in striated muscle cells: 1) The postsynaptic membrane of the skeletal neuromuscular junction 2) The membranes of the excitation-contraction coupling system, specifically the transverse tubules and sarcoplasmic reticulum that form the triad junction.

We developed a mammalian spinal cord neuron-skeletal muscle culture system in which neurites induce postsynaptic-like accumulation of acetylcholine receptors on the myotubes, from as early as 6 hours of coculture. We have shown that this inductive ability is predominantly a property of developing axons, as opposed to dendrites, and that the axon-myotube contacts can develop accumulations of synaptic vesicles, a typical synaptic cleft and a differentiated postsynaptic cell surface, as in the neuromuscular junction developing in vivo. We have devised a procedure to label the neurons in the cocultures with a fluorescent membrane dye in order to simultaneously observe the interactions betwen axons and myotubes and the resulting accumulation of acetylcholine receptors. We will use this culture system to investigate the role of agrin and other putative signals in postsynaptic receptor aggregation and to study other mechanisms of synaptogenesis.

We have been correlating data obtained by calcium imaging, immunocytochemistry and electron microscopy on cultured skeletal myotubes to determine the steps in the assembly of functional triads. Our results suggest that the structural and molecular differentiation of the membrane domains of transverse tubules and sarcoplasmic reticulum involved in excitation-contraction coupling occurs in concert with the formation of functional junctions between the two membrane systems. While the initial formation of these junctions is independent of association with myofibrils, we find that reorganization into proper triads occurs as the junctions become associated with the A-I border of the sarcomere.

Programmed Cell Death. During embryonic development, normal cell turnover, and many other physiological and pathological processes, cells die by the activation of an internally programmed mechanism termed apoptosis or programmed cell death. This process appears to be under genetic and biochemical control, but little is known about the relevant genes and mechanisms. Low-dose ionizing irradiation of pregnant rats of 16-18-day gestation causes the rapid and massive death of proliferating and differentiating stem cells of the fetal forebrain. We are examining this system (a) to determine whether these cells die by apoptosis and (b) to study the dynamics of mRNAs coding for gene products potentially relevant to apoptosis. We found that gamma-radiation elicits within 3 hours nuclear pyknosis and fragmentation in undifferentiated cells in the cortical neuroepithelium and also elicits fragmentation of fetal brain DNA into an oligonucleosomal ladder pattern characteristic of apoptosis. This DNA fragmentation requires ongoing RNA and protein synthesis, as determined by the use of specific inhibitors. These characteristics suggest that radiation kills these cells by the mechanism of

apoptosis. Northern blot analysis revealed that the abundances of most mRNAs tested (relative to total RNA), including those coding for p53, c-Myc, Bcl-2, and three housekeeping proteins, declined to 50-60% of the control levels by 3-5 hours after irradiation. In contrast, the mRNA for c-Fos, an early-response transcription factor, was increased to 20 times the control by irradiation. To identify novel induced genes induced by radiation, we prepared a subtractive cDNA library enriched in transcripts increased in irradiated fetal brain. Many clones were sequenced and found to represent transcripts only modestly increased in abundance (2-4-fold) or not decreased by irradiation. These results do not provide evidence for a requirement for dramatic induction of putative "cell death genes" in radiation-induced apoptosis. In another study, we also found that derangements of cellular protein phosphorylation by inhibitors of protein kinases (C and tyrosine-specific) or inhibitors of protein phosphatases elicit rapid apoptosis and oligosomal DNA fragmentation in P815 mouse mastocytoma cells.

Characterization of the HoxA7 Gene. The previous work on the HoxA7 gene has been extended by the assignment of the position of the transcription start site to be 1168 bp upstream of the translation start site. Transient transfection studies using NIH 3T3 cells using CAT reporter constructs provided evidence for the presence of both positive and negative regulatory elements in the upstream region.

Site-specific Mutagenisis of Presumptive ATP Binding Sites in E. coli Adenylate Cyclase. Further mutagenesis studies of the region around lysine-196 revealed the essential nature of the R188-K196 region. A model was developed for a unique ATP-binding motif in which there is a repetition of three basic residues on one face of an amphipathic helix that interacts with the three phosphate groups of ATP.

Requirement of Sugar Transport Proteins for Stimulation of Adenylate Cyclase Activity by Nucleotides. The allosteric stimulatory effect of nucleotides on phosphoenolpyruvate:sugar phosphotransferase system. The site of interaction of GTP with the adenylate cyclase complex was localized by photoaffinity labeling studies, which showed that the sugar transport protein HPr associated with other sugar transport proteins.

Dicistronic ptsI-crr Operon in Mycoplasma. The region of the Mycoplasma capricolum genome encompassing the genes for the sugar transport proteins Enzyme I and IIa^{glc} was sequenced. The data showed a unique dicistronic operon arrangement. The promoter and transcription start sites for these genes were determined.

The HPr Protein from Mycoplasma. The gene for HPr from Mycoplasma was cloned into an expression vector. The protein was highly expressed and purified to homogeneity. The protein was crystallized and the structure is being deduced by x-ray diffraction experiments.

The Enzyme I Protein from E. coli. Enzyme I was degraded with proteolytic enzymes to recover protease-resistant domains; the structures were determined by sequencing and mass spectroscopy. The gene for the core was cloned into an expression vector and the protein was expressed and purified to homogeneity; then the protein was crystallized for structural studies.

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Section on Enzymes

(1) Nitration of glutamine synthetase. The glutamine synthetase activity of *E. coli* is controlled by cyclic adenylylation and deadenylylation of a critical tyrosine residue in each subunit. Nitration of one tyrosine residue per subunit of unadenylylated enzyme by peroxynitrite led to changes similar to those provoked by adenylylation of the enzyme. Because peroxynitrite can be produced *in vivo* by reaction of nitric oxide with superoxide anion and because the nitration of tyrosine residues is irreversible, these results serve notice that nitration of proteins whose activities are under strict control by phosphorylation-dephosphorylation of tyrosine residues can seriously compromise their regulatory properties.

(2) Mechanism of action of the thiol-specific antioxidant enzyme. The thiol-specific antioxidant protein (TSA), found in yeast and most mammalian tissues, protects enzymes from inactivation by free radicals generated in a metal-catalyzed oxidation (MCO) system comprised of dithiothreitol (DTT), ferric iron, and oxygen. It was shown that TSA possesses thiol peroxidase activity that catalyzes the decomposition of hydrogen peroxide produced by the DTT-MCO system, and thereby prevents the generation of highly reactive radical species responsible for inactivation of enzymes. The specificity for protection against reactive species generated by the DTT-MCO system, but not by an ascorbate-MCO system, was explained by the observation that DTT, but not ascorbate, can reduce disulfide bonds in TSA and thereby convert it to a catalytically active form.

(3) Protein modification by 4-hydroxynonenal. 4-Hydroxynonenal (HNE) is a highly toxic oxidation product of polyunsaturated fatty acids. The cytotoxicity derives in part from reactions in which lysine, cysteine, and histidine residues of proteins add to the double bond of HNE to form Michael addition complexes. In continuing studies, it was shown that the aldehyde groups of the primary HNE-protein conjugates undergo secondary reactions with lysine residues of proteins to form stable Schiff base inter- and intra-protein crosslinked aggregates. These aggregates undergo further conversion to highly fluorescent compounds with characteristics similar to that of lipofuscin, which is known to accumulate in cells during aging.

(4) Degradation of oxidized proteins by the multicatalytic protease. The age-related accumulation of oxidized, catalytically inactive forms of enzymes is due in part to a decrease in ability of the multicatalytic protease (MCP) to catalyze the degradation of damaged proteins. It is now shown that the age-associated loss in protease activity is due to a decrease in peptidyl glutamyl peptide hydrolyase activity, which is one of the 3 different protease activities associated with the MCP complex. That the age-related loss of protease activity is due also to the accumulation of protease inhibitors is supported by the finding that HNE-generated protein-protein aggregates are not only resistant to proteolytic degradation by MCP, but they also inhibit competitively the ability of MCP to degrade the oxidized forms of other proteins.

Protein Ubiquitination. Protein ubiquitination has been implicated in numerous intracellular processes, such as protein turnover and cell cycles, etc. We showed that protein ubiquitination can be regulated by reversible phosphorylation. In addition to the kinases reported earlier, we purified a novel serine/threonine kinase, which is specific for E2-20kDa phosphorylation, from HeLa cells. Gel filtration indicates a MW of about 300 kDa and SDS-PAGE data suggest that the kinase may consist of 3 types of subunits. The stoichiometry is 0.45 mole phosphate incorporation per mole of E2 and phosphorylation enhances about 60% of the E2-20kDa activity to ubiquitinate histone H2A. Transcription factors fos and jun were found to be multi-ubiquitinated. E2-20kDa-catalyzed, multiubiquitination of jun proceeds via a stepwise dissociative mechanism, while polyubiquitination catalyzed by E2-14kDa and E3 proceeds via a processive mechanism in which ubiquitinated jun remains bound to E3.

Cytosolic Ca(II) Oscillation. In response to external stimulants, many cells release Ca(II) in an oscillating manner. In HeLa cells, we found that calmodulin-dependent kinase II (CaMK II) and a calyculin A-inhibitable phosphatase play key roles in sustaining Ca(II) oscillation and regulating its frequency. Evidence for *in vivo* phosphorylation of inositol trisphosphate receptor has been obtained. A theory for interpreting Ca(II) oscillations in HeLa cells has been proposed.

Mg(II)-dependent, Ca(II)-inhibited Phosphatase. The structure and distribution of a novel Mg-dependent, Ca-inhibitable protein phosphatase (CIPase) from bovine brain has been studied. Partial cDNA sequence of CIPase reveals that it is akin to another Mg-dependent, type 2C protein phosphotase (PP2C). CIPase activity was found in every mouse organ examined and its amount was about twice as much as PP2C, indicating that it is a major serine/threonine phosphatase.

EPR Study of Free Radicals in Biology. Electron paramagnetic resonance spectroscopy and spin-trapping methods were used to identify and monitor the formation and utilization of free radicals. We found that: (a) when NCB-20 cells were subjected to oxidative stress, glutathionyl radicals were formed inside the cells. The glutathionyl radical signal was not observed when the cells were pretreated with N-acetyl-L-cysteine (NAC), which can protect the antioxidant enzymes from oxidative damage. The time course shows enhanced $GS^{\bullet}$ formation, observed only in the absence of NAC, after a lag phase consistent with the idea that the majority of $GS^{\bullet}$ was generated after the antioxidant enzymes failed to function. (b) α-Ketoaldehyde is involved in glycation of proteins and nucleic acids. To investigate these reactions, we studied the reaction between methylglyoxal and amino acids as a model system. It was found that a stable free radical intermediate was formed during the crosslinking reaction. This intermediate was identified as a cation radical in which the amino groups of 2 amino acids are crosslinked by 2 carbonyl groups of a methylglyoxal molecule.

Mechanisms of Electroporation. Using probe molecules, we found electroporation leads to the formation of asymmetric pores on both sides of the membrane-facing electrodes with small pores and higher population on the anode side and large pores and lower population on the cathode side. The asymmetric transport pattern is neither caused by electrophoresis nor is it due to one-sided membrane breakdown as previously believed.

Role of Fe in Calcineurin Catalysis. Calcineurin, a calmodulin-dependent phosphatase previously identified as Zn and Fe-containing enzyme in this laboratory, was found by EPR and activity studies to require Fe(II) for activity. The Fe(III) form of calcineurin yielded EPR signal at a g factor of 4.3 and the line shapes are typical of the tetrahedral coordinates found in transferrin. But the Fe(III) enzyme form was inactive with p-nitrophenylphosphate as substrate.

Section on Protein Function in Disease

Research in this section focusses on oxidative modification of proteins, a covalent modification which has been implicated in important physiologic and pathologic processes. Research goals include determination of the chemical and structural changes induced by oxidation; identification of the processes affected by oxidative modification; purification and characterization of the systems which catalyze the modification and subsequent proteolysis; understanding the controls which may regulate the modification and proteolysis of specific proteins; and application of this knowledge to the rational design of irreversible enzyme inhibitors. In the last year, emphasis was placed in three areas: (1) the occurrence of oxidative modification of proteins during aging; (2) detection and quantitation of oxidatively modified proteins; and (3) oxidative modification of the protease from the human immunodeficiency virus.

Several laboratories have established an increased total burden of oxidatively modified proteins during the aging process. Utilizing electrophoresis and isoelectric focussing we noted the presence of a highly oxidatively modified protein in liver extracts from young, male rats. This protein virtually disappears in extracts from old rats, leading to the hypothesis that its loss may be related to the increased oxidation of other proteins during aging. The protein was purified and identified as carbonic anhydrase, isozyme III. The purified protein from young rats carries a high oxidative burden, assessed by its carbonyl content of ~10 nmol carbonyl/mg protein. It is also variably glutathionylated. These findings suggest a possible role for carbonic anhydrase in defense against oxidative stress.

Assessment of oxidative modification requires convenient assays, and progress has continued in their development. We have established that metal-catalyzed oxidation of glutamine synthetase converts His-269 into 2-oxo-histidine, providing a specific marker for oxidative modification. It was detected by both mass spectral analysis of an HPLC peptide map and by routine Edman sequencing of the modified peptide. Also, immunodetection of carbonyl-containing proteins has been successfully extended to both electrophoresis and isoelectric focussing of tissue extracts.

Replication of the human immunodeficiency virus and progression of acquired immunodeficiency syndrome has been linked to oxidative stress. We have therefore focussed on the effects of oxidative modification of the HIV protease, having shown earlier that the two cysteines of the protease are particularly susceptible to oxidation. Utilizing mutants containing only one cysteine residue we demonstrated that mixed disulfide formation occurs readily with either residue. Reaction with Cys-95 completely inactivated the protease, presumably because of its location at the dimer interface. However, reaction with Cys-67 also reduced activity by 50%, a somewhat surprising result because this residue is located on the surface, well-removed from regions known to be functionally important. Reversible oxidation of either or both residues may be important in regulation of protease activity and of HIV replication.

In humans and animals the trace element selenium (Se) is a key component of certain essential enzymes that control development and protect tissues from reactive organic peroxides. Proof that these enzymes contain Se in the form of selenocysteine was based on earlier chemical studies in this laboratory on the selenoprotein A component of clostridial glycine reductase. Other Se enzymes in prokaryotes include hydrogenases, formate dehydrogenases, xanthine dehydrogenase and nicotinic acid hydroxylase. The latter two contain Se in a dissociable cofactor instead of in selenocysteine. Recent EPR studies with Se enzymes that also contain molybdopterin show that Se is coordinated to Mo in the substrate site; in formate dehydrogenase it is the Se of SeCys and in nicotinic acid hydroxylase it is the Se of this unidentified cofactor that is involved in protein based radical species.

In prokaryotes an oxygen labile reactive Se compound, selenophosphate, is the biological Se donor for specific SeCys incorporation into proteins and for synthesis of 2-selenouridine residues in tRNAs. Using antibodies elicited to *E. coli* selenophosphate synthetase, the enzyme was detected in *Methanococcus vannielii* extracts and partially purified from this source. Cross reactivity with a protein in rat brain extracts suggested that SeP also serves as Se donor in eukaryotes. Using wild type and mutant forms of *E. coli* selenophosphate synthetase, amino acids essential for catalytic activity and MnATP binding have been identified. Both Mg and potassium are required for enzyme activity; Zn is inhibitory. The mechanism of SeP synthesis is thought to involve an initial reaction with ATP to form Enz-P-P with liberation of AMP followed by reaction of Enz-P-P with NaSeH to generate SeP and Pi. Contamination of selenophosphate synthetase preparations with trace amounts of adenylate kinase interfered with detailed mechanism studies. For removal of the contaminant, adenylate kinase was isolated and antibodies were elicited in sheep for preparation of an antibody affinity matrix.

A Se-dependent enzyme termed tRNA 2-selenourdine synthase was isolated (50-60% pure) from Salmonella and shown to convert 2-thiouridine in tRNAs to 2-selenouridine. No other activator is required for this sulfur replacement reaction. A direct attack of SeP on the C-S bond of the thiouridine is suggested.

An unusual quinone and dithiol dependent phosphatase that utilizes p-nitrophenylphosphate as sole known substrate was isolated earlier from *Clostridium stricklandii*. Antibodies were produced in rabbits and purified for use in screening experiments to provide clues as to possible role and distribution of the enzyme.

A cytotoxic aldehyde, 4-hydroxy-2-nonenal (HNE), produced by lipid peroxidation is an active protein cross-linking reagent. Using N-acetyllysine as a model compound, two of the products of the reaction of HNE with this amino acid were shown to be a Michael adduct and a Schiff base formed by reaction of the Michael adduct with a second molecule of N-acetyllysine. The latter compound accounts for the cross-linking activity. Similar products were identified in hydrolysates of glucose-6-phosphate dehydrogenase that had been treated with HNE. A third fluorescent product appears to be similar to lipofuscin, the pigment that is associated with aging.

Section on Protein Chemistry

The Section on Protein Chemistry is studying the physical and chemical properties of macromolecules of biological interest, processes of protein folding/unfolding, and the roles of ligand binding and inter- and intra-subunit interactions in enzyme catalysis and regulation. A goal of protein folding studies is to understand the nature of interactions that stabilize unique, biologically active three dimensional structures of proteins. A better understanding of the forces that govern protein folding will facilitate progress in many areas of biotechnology in which methods are being developed to produce proteins that can be applied as diagnostic and therapeutic agents in different diseases. For studying the folding/unfolding and assembly of complex, multisubunit proteins our group is using many physical-chemical methods. If an unfolding reaction can be described as a reversible, two-state transition, the thermodynamics of protein folding/unfolding can be determined experimentally from measuring the free energy of unfolding as a function of temperature. Differential scanning calorimetry (DSC) directly gives thermodynamic parameters and, in addition, provides some insight into the mechanism of unfolding of a biopolymer. Isothermal calorimetry is used also for studying interactions of proteins with ligands and other proteins. Spectral techniques are used to monitor changes in the environment of aromatic amino acid residues. Circular dichroism measurements show changes in secondary and tertiary structures. Ultracentrifugation, gel-filtration, and light scattering give information on changes in the size and shape of biopolymers.

Thermally induced reversible, partial unfolding transitions of dodecameric glutamine synthetase (622,000 M_r) from *E. coli* have been studied at pH 7. We have shown that each subunit has two domains of slightly differing stability ($\sim$2°C with a midpoint at 51.6°C) and that cooperative interactions link partial unfolding of all subunits within the dodecamer. However, the overall heat absorbed in these reversible transitions is $\sim$4% of that estimated for complete unfolding (8 $\pm$ cal/g).

Observations on the unfolding of the holo-tryptophan synthase $\alpha\beta\beta\alpha$ multi-enzyme complex (146,000 M_r) at pH 8 (with bound pyridoxal phosphate) from *S. typhimurium* are consistent with a model of six sequential two-state transitions for the $\alpha\beta\beta\alpha$ complex: 3 transitions at $<$ 65°C involve exposure of Trp 177 and an ellipticity decrease of bound cofactor in β chains prior to a sequential unfolding of α chains and 3 transitions at $>$ 7°C occur as Tyr residues are exposed and β chains unfold. At 90°C, a loss of $\sim$70% secondary structures of $\alpha\beta\beta\alpha$ is estimated. The cofactor increases both the linkage between unfolding domains and the stability of $\beta\beta$ subunits.

Thermally induced unfolding reactions in *Acanthamoeba* myosin II and rabbit skeletal muscle myosin at pH 7.5 have been characterized thermodynamically. We conclude that differences in the cooperative unfolding transitions of these myosins relate to rod structures and possibly also rod-head interactions. Binding of ATP markedly stabilizes head regions of both myosins.

Influenza virus hemagglutinin appears to adopt a molten globule conformation under fusogenic conditions (*i.e.*, pH 4.9, 37°C). From pH 7.5 to 5.0, a destabilization of tertiary structures and a stabilization of secondary structures is evident.

Ultracentrifugation has been used to determine ligand promoted shape and size changes of macromolecules and also for studies of interacting biopolymers. Dephospho- and phospho-myosin II from *Acanthamoeba*, *E. coli* glutamine synthetase, ClpA (± nucleotide) and ClpP of the *E. coli* ATP-dependent protease complex were studied.

Section on Signal Transduction

This section conducts research in two areas: (A) cell signaling involving phospholipases and (B) antioxidant enzymes.

(A) Cell proliferation, cell differentiation, and cell function are controlled by extracellular signal molecules that include hormones, neurotransmitters, cytokines, and growth factors. Some of the mechanisms by which these signals are transmitted across the cell membrane and transduced into complex cellular responses include the generation of second messengers from phospholipid by signal-activated phospholipases such as phospholipase C (PLC) and phospholipase D (PLD).

PLC, which hydrolyzes inositol-containing phospholipid and generates two second messenger molecules, inositol 1,4,5-trisphosphate and diacylglycerol, exists in at least 10 different isoforms in mammalian tissues. These isoforms can be divided into three types, PLC-β, PLC-γ, and PLC-δ. Previously, we have suggested that the diversity of PLC isoforms partly reflects different transducer molecules involved in their activation. The γ-type isozymes are activated when specific tyrosine residues of them become phosphorylated, whereas the β-type isozymes are activated by G protein α subunit Gqα as well as by G$\beta\gamma$ subunit. During the period covered, we found that the carboxyl-terminal region of PLC-β2 is required for activation by Gqα and that G$\beta\gamma$ subunits interact with a different (likely amino-terminal) region of the enzyme. This result suggests that two different receptors, one linked to a Gq protein and the other to a non-Gq G protein, can activate a single PLC-β molecule concurrently by generating Gqα and G$\beta\gamma$ subunits. the regulation of PLC-β4 by G proteins was also studied. Similar to the other three β-type (β1, β2, β3) isozymes, PLC-β4 was activated by Gαq. However, unlike the others, PLC-β4 was not responsive to activation by G$\beta\gamma$ subunits. PLC-β4 was not responsive to activation by G$\beta\gamma$ subunits. PLC-β4 was also shown to differ from the other three isozymes in that it is selectively inhibited by ribonucleotides.

PLD, which hydrolyzes phosphatidylcholine and generates choline and phosphatidic acid, itself a molecule with potential second messenger functions and a precursor of the second messenger molecule diacylglycerol, has not been purified from mammalian tissues. We embarked on the purification of PLD and demonstrated that rat brain membranes contain at least two different PLD activities, one dependent on oleate and the other on ADP-ribosylation factor. These two activities were partially purified.

(B) Hydroperoxides (H_2O_2 and ROOH) are damaging in living cells because they give rise to the radicals. The cellular machinery is equipped to limit the accumulation of hydroperoxides. We recently identified a previously unidentified peroxidase (Tpx). Tpx defines a family (named the peroxiredoxin family) of more than 26 different proteins which are present in organisms from all kingdoms, but have not been associated with known biochemical functions. All members of the peroxiredoxin family contain one conserved cysteine, which appears to be the primary site of reaction with hydroperoxide. The diversity in the amino acid sequences of the family members may in part reflect different mechanism involved in the regeneration of reduced peroxiredoxin.

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LABORATORY OF BIOPHYSICAL CHEMISTRY
CHEMICAL STRUCTURE SECTION
STRUCTURAL BIOPHYSICS SECTION
STRUCTURAL NUCLEAR MAGNETIC RESONANCE
. . . . . BIOPHYSICAL INSTRUMENTATION

The areas currently under study and development by the six senior members of the Laboratory include peptide, protein, and oligosaccharide mass spectrometry (Fales), small molecule high resolution x-ray crystallography,scanning tunnelling and force field microscopy (Silverton), nuclear magnetic resonance and computer modeling of proteins, and in vitro protein synthesis (Ferretti), nuclear magnetic resonance spectrometry of smaller molecules (Highet), biocalorimetry and near infrared spectroscopy (Berger), and countercurrent chromatography (Ito).

Ferretti continues his studies on peptides and small proteins in the free and bound states in an effort to understand conformation-activity relationships. Solution and solid state nuclear magnetic resonance (NMR) spectroscopy is being used along with computer approaches to calculate minimum energies, simulate annealing,and restrained molecular dynamics. Ancillary techniques are circular dichroism spectropolarimetry, mass spectrometry, and low angle neutron scattering. To obtain sufficient protein for these studies, he also continues efforts in continuous flow protein translation.

This year he has completed the three dimensional structure of the 77 amino acid homeodomain protein NK-2 from *Drosophila melanogaster* (synthesized in *E. coli*) along with a secondary structure and low resolution tertiary structure of the protein bound to its consensus DNA binding sequence. In the absence of DNA, a helix-turn-helix domin was identified, the last helix of which is comparatively short. Nonetheless, this protein has a high affinity for the duplex DNA, the recognition section doubles in length in its presence, and the protein becomes much more thermally stable. Analysis of the data suggests that mutating three nearby sites should alter the recognition without changing the binding constant and these proteins have recently been prepared.

In studies on the 828-848 intraviral domain of the HBX2 isolate of gp41, addition of SDS micelles, negatively charged phospholipid liposomes, or trifluoroethanol changes the protein from a random coil to an ensemble of ordered structures with up to 60% helical content. The suggestion is that the peptide backbone is in the water phase with part of the amino acid side chains penetrating the lipid water interface. Neutron reflectrometry experiments at NIST should confirm this structure.

The three dimensional structure of partially unfolded states of cytochrome c protein ISO2 from *Saccharomyces cerevisiae* is being studied with the aid of newly synthesized mutants. Assignments for approximately half of the amino acid residues have been made and a preliminary model of the unfolding pathway has been developed.

In other NMR work Highet has elucidated the structures of two Δ-substituted heme derivatives resulting from the action of a dihydropyridine on haemoglobin as found by Osawaga and Sugiyama of the Laboratory of Chemical Pharmacology. The hydroxyethyl group of one shows unusual assymetry. With Ito and Weisz (FDA), he has established the structure of several fluorescein food dye impurities and with Murphy(LCB), several phosphopeptides have been shown to be H-phosphoranes. With Rice and Ohshima (NIDDK) he has addressed the question of conformation related to the agonist/antagonist activity of certain optical antipodes. Finally, he is preparing a definitive review of the chemistry and pharmacology of the antimalarial drug artemisinin.

Silverton is interested in determining absolute and relative stereochemistry of compounds having anti-retroviral and enzyme inhibitory activity using x-ray crystallographic and atomic force microscopy techniques. This year he has investigated the tendency of chiral forms of thalidomide to assume achiral structures since, despite its deleterious effects on fetal development, it appears to have promise in AIDS therapy. In related work, he has solved the structure to an error of 1 ppm of the dextrorotatory form of the anti-AIDS compound cytallene. Structures of hormone inhibitors, coordination compounds, and an unusually large terpenoid compound have also been completed. In the area of atomic force microscopy, he is working with Agoston and Nirenberg on the structures of plasmids. The aim here is to check the validity of the dramatic micrographs reported elsewhere, since the data is so extensively processed.

Ito, extending his recent discovery of pH zone refining countercurrent chromatography, has successfully separated gram quantities of enantiomers of a leucine derivative using a chiral selector in the stationary phase. This technique will undoubtedly be of great interest to the pharmaceutical industry because of their interest in preparing pure enantiomers of chiral drugs. In other work, he has extended the technique to the displacement mode (rather than the usual reverse displacement mode). This offers the advantage of collecting fractions free of salt and the possibility of extending the method to ligand-affinity separations. He has also shown that the method works with bases as well as acids, separating several alkaloid mixtures and gram quantities of di- and tripeptide derivatives.

Berger, using his specially designed microcalorimeter, continues the study of the dependence of CO_2 binding to hemoglobin as a function of chloride concentration. He finds a "pK_{Cl}" of approximately the same as the physiological whole blood

concentration of chloride, suggesting that chloride plays an active role in CO_2 transport. He suggests that the amino terminal valine, forming carbamates with CO_2 or binding to chloride is the source of this interaction.

In other work, he continues his studies of the analysis of the kinetics of ATP hydrolysis by Na-K AtPase using near infrared spectroscopy. As mentioned last year, using static mixtures, he has calibrated his system for ATP, ADP, and P_i in the 1-25 micromolar region with < 5% error using six variables for the best fit. Hydrolysis of ATP in the 10 second time range was then monitored and, while the overall density agrees with an independent assay of inorganic phosphate, the complex spectral data was not yet able to be analyzed in terms of its components.

Fales, in core work, continues his investigations of the ion trap as an especially simple and unique mass spectrometer. Modifications to allow increased mass range and resolution have finally been completed and with the assistance of Visiting Fellow Kate Zhang, he is in the process of rebuilding it for electrospray analysis of proteins.

The matrix assisted laser desorption (MALDI) mass spectrometer run by E. Sokoloski has been operational in the Laboratory for 6 months and, as predicted, has solved a multitude of problems. Its sensitivity is one or two orders higher than the electrospray method and it is far less sensitive to impurities and buffers. Its resolution is lower however, and it has not yet been proved to be capable of fragmentation (sequence) analysis via its reflectron. The current mass spectrometric method of choice is to run an unknown peptide or protein first on MALDI and if successful, repeat its analysis on electrospray for higher accuracy and posible fragmentation analysis. Using one or both of these techniques, problems have been investigated from a wide group of researchers: E. Stadtman, D. Davis, H. Kaiser, M. Vaughan, S. Tsai, R. Levine, H. Brezesca, L. Tsai, Y. Osawa, W. Leonard, Y. Kimura, J. Rade, E. Kwon, M. Knepper, all of NHLBI, Y. Lee, H. Krutzsch, E. Appella, P. Randazzo, R. Kahn, N. Vamvakopoulos, J. Berzofsky (NCI), P. Backlund, P. Pentchev (NIMH), H Jaffe, G. Ehrenstein (NINDS) Y. Lee (NICHD), F. Robey, J. Thompson (NIDR), A. Liotta, J. Roth, J. Pitha (NIA), L. Pannell, P. Lecchi, A. Basil, W. Simmonds (NIDDK), J. Coligan (NIAID), T. Duncan (NEI). Outside collaborators include K. Schaffner (Waksman Institute), P. Watkins, (Johns Hopkins), and W. Roelofs (Cornell). Many of these are relatively short range studies involving a few samples, while others (Kahn, Vamvakopoulos, Jaffe, Thompson, Vaughan, Liotta, Rade) involve considerable reanalysis and interpretation.

Zhang and Fales have also undertaken with M. Potter (NCI) an analysis of the silicone implants used in breast reconstruction. Low molecular weight monomers in these implants have recently been found to cause formation of plasmacytomas in susceptible mice and they are seeking the responsible agent. Fales is also

working with W. Gahl (NICHD) to ascertain the cause of the increase in polyprenoids in the urine of children with Hermansky-Pudlak albinism, and an 85 carbon dolichol has been identified.

Annual Report of the Laboratory of Cardiac Energetics

National Heart, Lung and Blood Institute
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The major goal of the Laboratory of Cardiac Energetics is to improve our understanding of the cellular and molecular processes involved in the conversion of energy to useful forms of work in the heart and other tissues. With this insight we hope to develop new strategies for the diagnosis, prevention and treatment of heart disease. Our technological approach to these problems is the use of non-invasive nuclear magnetic resonance (NMR) and optical spectroscopy techniques. These methods permit the non-invasive monitoring of several critical parameters of energy metabolism including metabolites, blood flow and tissue oxygenation in intact tissues or humans. The application of these technologies to humans allows us to evaluate these tools as non-invasive diagnostic modalities.

The major energy metabolism pathway we have been concentrating on over the last year is the complex interaction between energy conversion processes in the heart (i.e. oxidative phosphorylation), muscle contraction (i.e. pumping of blood) and coronary blood flow. For the heart to function properly these three elements must be orchestrated with remarkable accuracy to provide the appropriate amount of oxygen, substrates and energy in the appropriate form to support the pumping of the blood. Myocardial muscle contraction is believed to occur by utilizing the energy in adenosine triphosphate (ATP) produced predominately by oxidative phosphorylation occurring in the mitochondria and converting oxygen to water. To use ATP for muscle contraction, ATP is hydrolysed to adenosine diphosphate (ADP) and inorganic phosphate (Pi). It was long believed that these hydrolysis products of ATP serve as the intracellular signals which regulate the rate of oxidative phosphorylation in the heart. While the further breakdown of ADP to adenosine was believed to regulate the resistance of the coronary blood vessels and thereby control the coronary blood flow. However, as we have previously demonstrated, the hydrolysis products of ATP, ADP and Pi, do not change during physiological increases in work. That is, under conditions where the turnover of ATP has increased almost 5 fold the hydrolysis products of this reaction do not increase. This indicates that the ATP, ADP and Pi levels are highly buffered by oxidative phosphorylation in the healthy myocardium and that these metabolites are unlikely to play an important role in the orchestration of metabolism or coronary blood flow. Hence some other cytosolic parameters must be responsible for the orchestration of these critical processes in the heart. Over the last year we have evaluated new sites where this regulatory processes could be occurring in the control of energy metabolism during work stress.

Previous studies on the control of oxidative phosphorylation in the intact heart suggested that the utilization of substrates may be a rate limiting step. Indeed, we had proposed that the level of mitochondrial NADH, generated by substrate oxidation, may be a key controlling step in the regulation of oxidative phosphorylation. To evaluate this hypothesis further, we investigated the effects NADH on the kinetics of oxidative phosphorylation in

isolated mitochondria. Our previous studies revealed that the level of mitochondrial NADH is apparently a strong regulator of the maximum velocity (Vmax) of oxidative phosphorylation. The relationship between [NADH] and Vmax was essentially linear with a slope of approximately 2. However, over the last year using mitochondrial uncouplers, which remove the inhibition of oxygen consumption by the obligatory formation of ATP from ADP and Pi by F1-ATPase, we have discovered that the actual concentration of NADH is not rate limiting, it is apparently the flux capacity of the dehydrogenases which is more important in determining the Vmax of the mitochondria. These studies suggested that the NADH level is only reporting the electrochemical driving force across the mitochondrial membrane and its absolute concentration has little effect on the overall kinetics of oxidative phosphorylation. This hypothesis suggests that NADH is reporting the force driving oxidative phosphorylation and not significantly involved in the kinetic control. This conclusion is consistent with the linear relation between NADH and respiratory rate and the lack of co-operativity between NADH and ADP found previously.

In the course of these studies we became interested in the potential regulation of F1-ATPase by substrates and the electrochemical driving force. Recent studies from Harris et al. have suggested that this enzyme may be appropriately regulated to participate in the control of oxidative phosphorylation in cultured myocytes. Using data collected on isolated mitochondria and the heart, in vivo we have reached the conclusion that the F1-ATPase maybe an important regulatory site in the mitochondria. Our data, which show increases in F1-ATPase activity in response to substrate availability and workload, suggests that the F1-ATPase may be the major regulator of the maximum velocity potential of the mitochondria when the system is working at intermediate rates of respiration as occur in the intact cell. Our hypothesis is that the F1-ATPase is regulated to control the dissipation of the electrochemical gradient across the mitochondrial membrane. This hypothesis is now being tested in the laboratory using ion selective electrodes to follow the mitochondrial membrane potential simultaneously with the NADH levels and F1-ATPase activity.

Adenosine is a potent vasodilator that has been proposed as the feedback regulator between metabolic rate and blood flow. This hypothesis is based on the idea that extracellular concentrations of adenosine increase with increases in ATP hydrolysis resulting in a vasodilation which is proportional to the work load. Several lines of evidence from our laboratory suggest that this is not an appropriate model. We have found that the ADP and ATP concentrations do not change during 5 fold increases in metabolic rate and coronary blood flow. This suggests that the substrates for adenosine formation are not increasing in the heart with work. Our most recent studies involve the use of an adenosine agonist, 8-phenyltheophylline, to block adenosine action and evaluate the effects on coronary flow regulation. These studies revealed using a variety of techniques, including 31P NMR and vascular sampling, that the inhibition of adenosine action did not cause a "functional ischemia" during increases in cardiac work. This suggests that the heart is completely capable of increasing coronary blood flow to the appropriate levels without adenosine receptors. These results suggest that adenosine does not play a significant or necessary role in the regulation of coronary flow with increases in work. In the course of these studies we did find that the adenosine blockade

did decrease the sensitivity of cardiac contractility to pacing and dobutamine. For dobutamine specifically, this resulted in a marked shift in the dose response curve. The reasons for this decreased sensitivity of the myocardium in response to adenosine blockade is currently under investigation, as well as the investigation of other potential cytosolic and tissue level transducing systems between coronary blood flow and work. Our major efforts over the next year will involve the evaluation of extracellular K as a extracellular signal regulation coronary blood flow. This hypothesis is the result of our previous studies demonstrating a strong dependence of coronary resistance on ATP sensitive K-channels.

Dr. Laughlin has been evaluating the effects of different substrates and hormones on the rate of glycogen synthesis, measured using 13C NMR, in the canine heart with the goal of a better understanding of the mechanisms involved in substrate utilization in the intact heart. A surprising result in her studies was the discovery that infusions of lactate, pyruvate or ketone bodies to the local cardiac vasculature or systemically resulted in a large stimulation of glycogen synthesis. The degree of stimulation was larger than that attained with insulin infusions under identical conditions. Thus, cardiac glycogen synthesis is extremely sensitive to circulating substrate concentrations. To further these observations, we have recently shown that circulating substrates can also alter glycogen synthesis in rat skeletal muscle using similar techniques. These studies also followed the cytosolic glycerol-6-phosphate (G-6-P) levels in the rat and found that the stimulation of glycogen synthesis is correlated with an increase in G-6-P in both skeletal muscle and heart. This latter result supports our original hypothesis that alternate substrates increase glycogen synthesis by reducing the competition for G-6-P in glycolysis by providing alternate substrates for oxidative phosphorylation. These data on skeletal muscle suggest that substrate competition is an important factor in whole body glucose storage mechanisms and not just cardiac levels as suggested in our previous studies. The demonstration that this effect is linked to G-6-P levels and establishing the role of the skeletal muscle completes the objectives in this overall program on glycogen synthesis.

We have been evaluating the effect of chronic muscle pacing on the metabolic properties of in vivo skeletal muscle. The transformation which occurs is believed to convert skeletal muscle energetics into a pattern which mimics cardiac muscle. In this model, skeletal muscle is chronically stimulated in vivo for several weeks. After this period a transformation occurs in which the muscle takes on many of the characteristics of cardiac muscle. We have begun to evaluate this system to take advantage of this transformation to gain information on the potential metabolic control sites in the normal heart. Dr. T. Ryschon has developed an in vivo rabbit TA/EDL muscle preparation for this purpose. We have demonstrated that a appropriate transformation of this muscle occurs with chronic pacing and that a metabolic pattern exists similar to the heart with regard to a work stress challenge. Our progress on this project was slowed by technical difficulties in obtaining the micro-stimulators required for this preparation. However, this roadblock has been overcome by the discovery of a commercial source. Studies are now underway to evaluate the effects of work stress the metabolic rate in this tissue using a newly developed blood flow measurement technique based on fluorescent microspheres. This

will then be followed by studies on the NADH and Ca handling in the tissue. Hopefully, this preparation will not only teach us about the transformation of skeletal muscle by training, but also provide important insights into the regulation of oxidative phosphorylation in the heart.

A large portion our of efforts over the last year have been devoted to the development of NMR techniques to the study of organ physiology and biochemical structure in vivo. These approaches are being developed to provide new tools in studying the function of the body in vivo, as well as potentially provide new non-invasive diagnostic tools to clinically evaluate humans.
Using a saturation transfer approach, in combination with standard magnetic resonance imaging (MRI) techniques, we have successfully imaged the rate of magnetization transfer between various macromolcules protons and protons in water in intact tissues. The contrast generated by this process, termed magnetization transfer contrast (MTC), is unique in magnetic resonance imaging and is currently being developed for clinical applications around the world. These applications include the study of multiple sclerosis, blood vessel angiography, AIDS, cancer and sports medicine. This approach has also provided a unique insight into the basic mechanisms of water proton relaxation in biological tissues which we have been concentrating on over the last year.
In our attempts to evaluate the molecular mechanisms responsible for the MTC effect, we have studied a large series of macromolecules and lipids. In summary, these studies have demonstrated that a surface hydroxyl group is the most effective surface group in generating this effect while amine groups can provide a weak effect. Our most recent studies involve the use of specifically targeted deuterium labeling in lipids which effectively isolates different motional domains in the lipid system by preventing a given region to communicate with the rest of the macromolecule due to 2-H inability to exchange magnetization via spin diffusion with 1-H. These studies have revealed that there are different region domains in the lipid bilayer system and that all of these contribute to magnetization transfer in the native lipid. These results also demonstrate that the overall magnetization transfer spectrum is a sum of all of the motional domains in the macromolecule and do not reflect the actual dynamics of the interaction site alone. Currently we are trying to use this new strategy in the isolation of the water-lipid interaction site in lipid systems. These in vitro studies are critical in the interpretation of numerous in vivo results on the differential effects of water/macromolecule magnetization transfer in health and disease.

Since November, 1991 the Laboratory has been outfitting a 4 Tesla 1 meter bore NMR system for human studies. This is the highest magnetic field strength available for whole body NMR studies. Only 2 other similar systems are present in the world. This high field has demonstrated that it will improved signal to noise in spectroscopic studies of tissue biochemistry (as much as 3 fold) as well as improve the spatial (3 fold) and time resolution (7 fold) of NMR imaging studies. These advantages have made many new studies possible on humans which could not be performed at the more commonly available fields of 1.5 or 2 Tesla.

In the last year there have been several major technical accomplishments. We have

established the contribution of different loss mechanisms associated with making NMR measurements at 170 Mhz in man. Using several coil and phantom(models of human structures) we have established that radiation and dielectric losses can be minimized while the major loss mechanism is the inductive losses induced by both conductive and dielectric currents in the body. Using this information we are currently designing new coil configurations to minimize the dielectric currents. In addition, coils using the dielectric current rather that a conductive current have been constructed and demonstrated.

In our studies of the heart, we have been exploiting a new coil arrangement using two opposing coils. This has permitted excellent radiofrequency penetration characteristics which surpass previous designs. However, the detection of EKG and associated large motion artifacts in the body at 4T have limited the applications to-date. Our studies using retrospective gating have failed, due to the inadequate coverage of k-space in a reasonable period of time. Thus, we have focused our efforts on fast imaging techniques. This has resulted in the generation of a new gradient set designed specifically for the heart and implementation of several new fast imaging techniques. It is hoped that the combination of faster and more powerful gradients with fast imaging techniques will result in the successful application of high field NMR studies in the human heart.

We have begun a large series of studies on the metabolism and function of human skeletal muscle after the development of an exercise device which functions in the 4T magnet. This system has permitted us to evaluate the relationship between blood determinations of free and intracellular Mg levels with direct tissue measures using 31P NMR together with investigators in the NIMH and Clinical Center. No correlation was found suggesting that the typical clinical measures of blood Mg levels are not reflecting free intracellular levels. In addition, together with the Cardiology Branch of the NHLBI, we have begun to evaluate the effects of specific myosin genetic modifications on skeletal muscle energetics in vivo. These studies were conducted using 31P NMR to follow the high energy phosphates in patients during carefully controlled muscle exercise in the 4T magnet. These studies have suggested that specific alterations in myosin are also reflected in metabolic deficiencies in the soleus muscle. Based on the rate of creatine phosphate depletion and re-synthesis in exercise. We are currently generating new hypothesis in establishing why a genetic modification of myosin results in an apparent metabolic compromise of this skeletal muscle.

Another major project in the LCE is the use of MRI techniques in the evaluation of tissue oxygen levels and blood flow. These studies deal with the development of methods for non-invasive monitoring of perfusion and blood oxygenation, and real-time imaging. The basic experimental tool is the use of Echo-Planar Imaging (EPI), a type of magnetic resonance imaging which acquires images in 0.1 sec. or less, much faster than conventional MRI. The images can be sensitized to spatial variations in blood flow and blood oxygenation, and can be obtained at a rate of up to 1 per second. Other imaging techniques such as Gradient Recalled Echo (GRE), Spiral, and Spin Echo have also been implemented.

In cardiac studies we are utilizing a canine heart model to evaluate the use of GRE to

monitor coronary blood flow and tissue oxygenation. Coronary blood flow alters the apparent spin lattice relaxation time (T1) of the tissue water while changes in hemoglobin oxygenation alters the apparent spin-spin relaxation time (T2). In the course of these studies, we have determined that blood volume measurements are critical for quantitating the effects observed in heart. Thus, we are developing new methods of determining cardiac blood volume using MRI.

The ability to map brain metabolic responses also relies on these NMR relaxation consequences of blood flow and tissue oxygenation. Using this approach the location and time course of metabolic alterations in the brain in response to stimuli can be assessed with mm spatial and sub-second temporal resolution. This is orders of magnitude better than previous techniques. The focus of these studies over the last year has been the evaluation of motor and visual processing in the brain as well as new efforts in pain and heat stimulation. A great deal of effort has been devoted to a better understanding of the physiological nature of the signals observed as well as a better understanding of the background "noise". We have established that the major noise sources in these experiments is the inflow of blood, and motion associated with the heart beat and respiratory action of the chest. The motor studies have progressed to the development of new stimuli protocols and the documentation of brain responses to real and imagined actions. The differences in these two providing important information on the processing of information within the brain. This has resulted in several neurological publications with our collaborators in several different Institutes on campus.

We have also attempted to monitor the metabolic responses of the spinal cord during both motor and heat/pain stimulation. These studies found no detectable MRI intensity changes due to T1 or T2 alterations in the spinal cord during stimulation. The reason for this result is unclear and we are currently following this phenomenon in animal models to establish the reasons for this lack of responsiveness.

Annual Report of the Laboratory of Cell Biology
National Heart, Lung, and Blood Institute
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The Laboratory organization remains as it was: Cellular Biochemistry and Ultrastructure Section, Edward D. Korn, Chief, M. Blair Bowers, J. Knutson, R. Chen and H. Brzeska-Bzdega; Molecular Cell Biology Section, J. A. Hammer, III, Chief and G. Jung; Cellular Physiology Section, E. Eisenberg, Chief, L. E. Greene; Membrane Enzymology Section, R. W. Hendler, Chief; Pulmonary and Cardiac Assist Devices Section, T. Kolobow, Chief. The Laboratory occupies approximately 9800 square feet in Building 3 and 2000 square feet in Building 10 with the use of animal surgery space in Building 14E. Major research interests continue to be: (1) the structure and function of non-muscle myosins, (2) the regulation of actin polymerization, (3) the structure and function of the 70-kDa heat shock proteins, (4) bioenergetics, (5) biological applications of fluorescence spectroscopy, (6) respiratory assist devices, and (7) membrane flow in eukaryotic cells. The following summary of major accomplishments in fiscal year 1994 is arranged by scientific topics rather than by organizational Sections.

Dictyostelium Myosins

Dr. Hammer had previously identified 6 putative myosin genes in *Dictyostelium*, in addition to the one myosin II gene and 5 myosin I genes that were already known. Several of these genes have been cloned and ~4 kb has been ~60% sequenced. The partial sequence is consistent with this gene coding for an unconventional myosin perhaps of the myosin V or myosin VI family.

The 5 *Dictyostelium* myosin I isoforms fall into two sub-families: the classic myosins I (myoB, myoC and myoD), that have the 3 tail domains (TH-1, TH-2 and TH-3) originally identified in the 3 *Acanthamoeba* myosins I, and the truncated myosins (myoA and myoE) that lack the TH-2 and TH-3 domains. Dr. Hammer and Dr. Yoshi Fukui (Northwestern University) have confirmed by immunofluorescence confocal microscopy the localizations of myoB and myoD to the actin-rich cortical regions which are the sites of active motile processes; myoB is the predominant isoform in filopodia. Incomplete studies show that myoC has a similar distribution as myoB and myoD. Studies *in vitro* with GST fusion proteins containing various regions of the tail domains of myoC have established that the glycine, proline-rich TH-2 domain contains the ATP-insensitive actin-binding site characteristic of the classic myosins I. A myoB mutant lacking the TH-3 domain (which resembles SH-3) has been introduced into myoB$^-$ cells and the protein shown not to localize at the leading edge of the amoebae as the wild type myoB does.

Efforts by Dr. Hammer and Dr. Goeh Jung to define the functions of the classic myosins I in *Dictyostelium* have thus far involved the preparation and study of a single myoB mutant, a double myoB/myoD mutant, and a triple myoB/myoD/myoC mutant. These mutants show progressive impairment in chemotactic streaming due, at least in part, to reduced rates of translocation which seems mostly to be due to myoB deletion. Doubling times and pinocytosis are also progressively reduced. When an integrated plasmid is utilized to express myoB in the myoB$^-$ cell line, it requires ~5-fold over-expression of normal levels to rescue the cells; expression at normal wild type levels does not alter the phenotype of the myoB$^-$ cells. In wild type cells, myoB is up-regulated ~10-

fold during early development whereas the plasmid myoB is not up-regulated in the rescued myoB$^-$ cells. This may be why 5-times normal expression levels are needed to rescue the myoB$^-$ cells.

Acanthamoeba Myosins

Previous work by Dr. Korn described two families of myosin in *Acanthamoeba castellanii*, one myosin II isoform (a classical filamentous myosin with two heavy chains and two pairs of light chains) and 3 non-filamentous myosin I isoforms (monomers with a single heavy chain). Dr. Hammer later demonstrated the existence of and partially purified a representative of a third family, referred to as a high-molecular-weight myosin I. Dr. Hammer has now developed a reproducible purification procedure for this myosin and obtained enough of the purified protein to characterize its properties. Its ATPase activity is enhanced ~30-fold by 45 μM F-actin and it has a Km for ATP of 15 μM; the enzyme translocates actin filaments at a rate of ~11 μm/s and appears to be unable to self-assemble into filaments; rotary shadowed electron microscopic images reveal a molecule with a single, large head and a single ~50 nm-long tail.

Dr. Korn and Dr. Ivan Baines have extended their studies on the *in situ* localization of total myosin IA, IB and IC to include the specific localizations of the phosphomyosins I, which are the enzymatically active forms. Essentially all of myosin IA is phosphorylated and, thus, the distribution of phosphomyosin IA is the same as total myosin IA (actin-rich cortex). Phosphomyosins IB and IC, however, are a small fraction of the total isoforms; phosphomyosin IB is relatively enriched in pseudopods while phosphomyosin IC - and F-actin - is 20-times more concentrated in contracting contractile vacuoles than in filling contractile vacuoles, providing additional support for the specific role of acto-phosphomyosin IC in contractile vacuole function.

The myosin I heavy chain kinase that phosphorylates and activates the *Acanthamoeba* myosins I is activated by autophosphorylation which, in turn, is enhanced by association of the kinase with acidic phospholipids and plasma membranes. Last year, Dr. Korn's group showed that the kinase can be fully activated simply by association with plasma membranes, prior to autophosphorylation. This year, Dr. Korn showed that the kinase can be fully activated by binding to phospholipid vesicles containing 15%-30% phosphatidylserine before there is significant autophosphorylation. Activation of kinase by either phospholipids or autophosphorylation involves an increase in Vmax with no change in Km for myosin I.

Dr. Brzeska-Bzdega has found that a 35-kDa, C-terminal fragment of the kinase has full, autophosphorylation-dependent activity which is lost by cleavage into 25-kDa (N-terminal) and 11-kDa (C-terminal) fragments. A cDNA clone corresponding to this region has been sequenced and shown to be highly homologous to the active domains of other protein kinases.

Actin Polymerization

Dr. Korn and Dr. Michael Bubb, in collaboration with scientists in NCI and at SUNY at Stony Brook, Health Science Center, have correlated the mechanisms of action of two cytotoxic natural products with their effects on actin

polymerization. Jasplakinolide, a cyclic peptide with a 15-carbon macrocyclic ring isolated from a marine sponge, was shown to bind to F-actin competitively with phalloidin with a Kd ~15 nM which is similar to its reported IC_{50} of 39 nM. As a result, it stabilizes actin filaments and causes G-actin to polymerize in an otherwise non-polymerization buffer. Unlike phalloidin, cells are highly permeable to jasplakinolide so it may be a useful pharmacological agent.

Another product of marine sponges, swinholide A, a 44-carbon ring macrolide consisting of two identical 22-carbon chains, was shown to be similarly toxic to cells in culture but by a contrasting mechanism. Cells round up, lose lamellipodia, filopodia and stress fibers and cytokinesis is blocked. *In vitro*, swinholide A severs and depolymerizes F-actin forming stable non-polymerizeable dimers, which it also does starting with monomers, with an apparent Kd ~25 nM for the interaction with actin dimer.

70-kDa Heat Shock Proteins and the Homologous Uncoating ATPase

Dr. Eisenberg and Dr, Greene have found that the uncoating of clathrin baskets by the bovine brain 70-kDa heat shock protein (hsp70) requires the presence of a 100-kDa cofactor protein which may be the previously described assembly protein, auxilin. The time course of uncoating shows an initial burst followed by a slow steady state. The ATPase activity of hsp70 also requires the presence of the 100-kDa cofactor and shows an initial burst and subsequent slower steady state just like uncoating. In the presence of excess cofactor, clathrin baskets activate hsp70 ATPase activity about 100-fold with a Kd of ~0.2 μM. The data stoichiometrically fit a model in which clathrin is in rapid equilibrium between baskets and hsp70 in its ATP form but dissociates from the baskets and binds tightly to hsp70 in its ADP form with subsequent conversion of ADP-hsp70 to ATP-hsp70 causing clathrin to dissociate.

A new 20-kDa assembly protein has been purified and identified as myelin basic protein (MBP). MBP-clathrin baskets are indistinguishable from baskets made with other assembly proteins and their dissociation by hsp70 similarly requires the 100-kDa cofactor.

Clathrin and certain peptides have been found to bind much more tightly to ADP-hsp70 than to ATP-hsp70 and the rate of clathrin dissociation has been shown not to correlate with the rate of ATP hydrolysis by hsp70. This supports the model stated above in which clathrin dissociation is not correlated with ATP hydrolysis but with replacement of ADP by ATP.

ADP-hsp70 is partially polymerized whereas ATP-hsp70 is monomeric. This is not, however, related to the different affinities of ADP-hsp70 and ATP-hsp70 for clathrin as Drs. Greene and Eisenberg have found that only the monomeric form of both state of hsp70 bind clathrin and that clathrin depolymerizes ADP-hsp70 by virtue of the fact that it binds specifically to the monomer.

DnaJ is an *E. coli* protein required for the interaction of DnaK, the bacterial hsp70, with various substrates. A yeast DnaJ interacts with yeast hsp70 with unknown functional consequences. It has now been found that, in the presence of ATP, yeast DnaJ causes yeast and brain hsp70 to polymerize. Polymerization is reversed when either ATP or DnaJ is removed. As the polymerized hsp70 does

not react with substrate, DnaJ interactions may be a method for shifting hsp70 into an inactive form *in vivo*.

Bioenergetics

Dr. Hendler continues to study the detailed mechanistic events of two primary proton pumps, bacteriorhodopsin and cytochrome oxidase. Bacteriorhodopsin is a trimer in the native bacterial purple membrane. Contrary to previous results of others, Hendler has been able to obtain much higher activity with purple membranes incorporated into liposomes than with monomers. In this reconstituted system, proton pumping by increasing the internal Pi content from 50 mM to 200 mM; the magnitude of protons pumped is equivalent to a decrease of ~3-4 pH units.

Bacteriorhodopsin participates in two different photocycles depending on the light intensity. Hendler has found that the light-dependent transitions are lost in the presence of concentrations of detergents too low to alter the trimeric structure of the bacteriorhodopsin, presumably by affecting on the lipid environment. The purple membrane contains about 10 lipid molecules per bacteriorhodopsin monomer, one nonpolar lipid and 9 polar lipid molecules. Preliminary data suggest that the nonpolar lipid is most affected by low concentrations of Triton X-100 that affect the photocycle.

Biological Application of Fluorescence Spectroscopy

Dr. Knutson continues to develop a laser-based facility and data analysis methods for time-resolved fluorescence spectroscopy of biomolecules to determine size, flexibility, folding and structural parameters of proteins, DNA and membranes that cannot be obtained by any other method. One set of studies involved the interaction of the homeodomain of the transcription factor oct-pou (and the coactivator VP16) with a series of DNA sequences. Knutson found that the tryptophan emission varied with the details of the DNA sequence, with greater changes within a class of DNA sequences than between classes, and that a tryptophan in the "activation face" of oct-pou remote from the DNA is affected differently by details of the DNA sequence. However, the overall shape and size of the complex were DNA-class dependent and independent of specific sequence details.

This technology has been successfully adapted to the detection of dense, colored or fluorescent objects within tissues with, in some cases, resolution of less than 1/50th of an inch for objects 1 inch deep in tissue. This method is now being used by others as a possible adjunct to mammography.

Respiratory Assist Devices

Dr. Kolobow belief that the pathological features associated with adult respiratory distress syndrome (ARDS) and acute respiratory failure (ARF) are, in large part, cause by the elevated pressures associated with mechanical ventilation, has gained considerable support over recent years. Dr. Kolobow continues to develop new procedures for performing mechanically assisted ventilation at normal inspiratory pressure. After inducing ARDS in a sheep model by mechanical ventilation at elevated pressure, Dr. Kolobow compared his new procedures to continued mechanical ventilation under elevated pressure.

The system employs intratracheal pulmonary ventilation utilizing an endotracheal tube (with a new laryngeal seal) newly designed by Dr. Kolobow and continuous positive airway pressure As little as 10%-20% of the lung is ventilated and the sheep controls both the respiratory rate and the tidal volume. This is unassisted, spontaneous ventilation with a greatly reduced dead space as the air or oxygen is delivered directly through the endotracheal tube thus bypassing the upper airways. All animals managed in the conventional manner died while all of the animals on the new system survived and were successfully weaned to normal ventilation.

ANNUAL REPORT OF
THE LABORATORY OF CELLULAR METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1993 through September 30, 1994

Research in the Laboratory of Cellular Metabolism has for several years been concentrated on guanine nucleotide-binding proteins that are critical control elements and signal transducers in cellular responses to the extracellular environment. Many of these are substrates for bacterial toxins that catalyze the ADP-ribosylation of specific amino acids and thereby alter protein activity. The properties and actions of some of these toxins have also been investigated, along with continuing attempts to identify and characterize analogous ADP-ribosyltransferases in animal cells, as well as the enzymes that remove the ADP-ribose and the proteins that are substrates in these kinds of potentially regulatory ADP-ribosylation cycles. Long term studies of specific cyclic nucleotide phosphodiesterases (PDEs) are now yielding considerable new information about a family of these enzymes that are critical sites of hormonal effects in many cells and are targets of several new drugs. In the past few years, much of our effort has been focussed on the 20-kDa ADP-ribosylation factors (ARFs) that are involved in intracellular vesicular transport in both exocytic and endocytic pathways. Studies have included cloning and expression to evaluate structure-function relationships and effects of post-translational modification as well as attempts to define interactions of individual ARFs with Golgi membranes in vitro.

1. Cyclic Nucleotide Phosphodiesterases (PDEs)

By degrading cAMP and cGMP, PDEs are critical determinants in the regulation of intracellular cyclic nucleotide concentrations. Six different PDE gene families have been identified and characterized. They differ in structural and biochemical properties, substrate affinities, sensitivities to specific inhibitors, and regulatory properties. For the past several years, this laboratory has focused on the "so-called" Type III cGMP-inhibited (cGI) PDE gene family. The cGI PDEs are characterized by high affinity for cAMP and cGMP, selective and specific inhibition by a number of positive inotropic agents, and rapid activation by insulin as well as by hormones that increase cAMP in adipocytes, hepatocytes, and platelets. They have been implicated in regulation of several important physiological processes, e.g., lipolysis, myocardial contractility, smooth muscle relaxation, and platelet aggregation.

We have for a number of years studied the rat adipocyte cGI PDE, which is phosphorylated and activated by insulin. This activation is important in the antilipolytic action of insulin. We have now cloned human and rat cDNAs encoding two distinct types of cGI PDEs referred to as cGIP1 (fat cell type) and cGIP2 PDEs. The open reading frames encode proteins of ~122 to 125 kDa. The cGIP1 and cGIP2 PDEs share a common domain organization pattern. The deduced amino acid sequences in the N-terminal portions of the proteins predict regulatory

domains, including hydrophobic putative membrane association domains and potential sites of phosphorylation by cAMP-dependent protein kinase. Results of collaborative studies indicate that, based on the deduced sequence of RcGIP1 PDE, serine 427 in rat adipocyte cGI PDE may be a site for phosphorylation by cAMP-dependent protein kinase *in vitro*.

The deduced amino acid sequences of RcGIP1 and HcGIP1 are similar, and differ from those of RcGIP2 and HcGIP2 which are also similar to each other. The deduced sequences of cGIP1 and cGIP2 PDEs differ primarily in the N-terminal portions of the proteins. In contrast, the deduced sequences of the conserved catalytic domains are very similar in cGIP1 and cGIP2 PDEs with the exception of an insertion of 44 amino acids, which is missing in the conserved domain of other PDE families and which differs in cGIP1 and cGIP2 PDEs. This region may be unique to the cGI PDE gene family and could represent a conserved domain specific for different cGI PDE subfamilies. The gene for HcGIP1 was localized on human chromosome 11, that for HcGIP2, on chromosome 12. Expression of RcGIP1 and RcGIP2 mRNAs differs in different tissues. For example, RcGIP1 mRNA is expressed in rat adipocytes and increases during differentiation of 3T3-L1 adipocytes, whereas RcGIP2 mRNA is most abundant in rat myocardium. Taken together, our results suggest that the cGI PDE gene family contains at least two subfamilies, products of different but related genes. cGIP1 PDEs (or cGIP2 PDEs) from rat and human are more closely related than cGIP1 and cGIP2 PDEs from the same species.

Full-length human cGIP2 PDEs and a truncated ~54-kDa protein lacking the N terminal region, which contains a hydrophobic putative membrane association domain, were expressed in Sf9 cells. Some of the properties of the recombinant cGIP2 PDEs were similar to those of cGI PDE that is associated with human myocardial microsomal fractions, i.e., high affinity for cAMP and sensitivity to inhibition by OPC 3911 or cGMP, but not Rolipram. The full-length form was recovered primarily in association with particulate fractions, whereas truncated activity was primarily cytosolic. After solubilization with a combination of salt and detergent, the full-length form was eluted from an HPLC gel filtration column as a >440,000-kDa aggregate/oligomer, whereas the truncated form behaved as a dimer. It was inferred that the hydrophobic domains in the N-terminal region may be important in membrane association and/or self association of cGI PDEs.

2. ADP-ribosylation factors: 20-kDa Guanine Nucleotide-binding Proteins

ARF (ADP-ribosylation Factor) was discovered in Gilman's laboratory as an activator of cholera toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$. ARFs have since been shown to participate in several types of intracellular vesicular transport. ARFs of similar size have been found in all eukaryotic species, but not in bacteria. By cDNA cloning, we identified six mammalian ARFs, which were grouped into three classes, based on size, deduced amino acid sequence, and phylogenetic analysis. Mammalian ARFs 1 and 3 (class 1) have been implicated in several pathways of intracellular membrane trafficking, but it has been difficult to obtain clues to the physiological actions of ARFs 4, 5, and 6. The *Drosophila melanogaster* counterparts of these ARFs were sought in the hope that they might be easier to study successfully in this organism, which has many experimental advantages, and

about which there is so much genetic and developmental information. This year we completed characterization of class II and class III ARF genes and proteins. Kahn had earlier described a class I ARF so that examples of each of the three mammalian classes have now been identified in *D. melanogaster*. The three ARFs were more similar to those of the same classes in other species than to each other, were located on different chromosomes, and were differently expressed in heads vs. legs vs. bodies, consistent with the conclusion that they function as independent entities. The identification of ARFs that appear to be the counterparts of each of the three mammalian ARF classes in simpler eukaryotes (e.g., *Drosophila*) may facilitate elucidation of their function(s) by providing experimental systems (organisms) that will be easier to work with.

We had earlier found that mRNAs for mammalian ARFs 1, and 3 appear to be products of alternative polyadenylation. In more recent studies, testis-specific expression of short forms of ARF 4 mRNA was observed in several species. Appearance of the short form during mouse development coincided with late spermatogenesis. Sequences of the 3´-untranslated regions of the rat and human genes were very similar with identical polyadenylation signals at comparable positions. Several testis-specific mRNAs shorter than corresponding somatic mRNAs have been reported. Shorter mRNAs, thought to be more stable than the longer forms, may compensate for the cessation of transcription at late stages of spermatogenesis. In rat testis, other ARF mRNAs have the same size as those detected in somatic cells. Why only ARF 4 mRNA is synthesized as a short form during spermatogenesis is an intriguing question.

Characterization of the ARF-toxin interaction may yield information that can be related to the interaction of ARF with its physiological effector. In addition, some studies provide new information about the structure and function of cholera toxin, which may well be applicable to several analogous toxin ADP-ribosyltransferases. LT (*E. coli* heat-labile enterotoxin) resembles cholera toxin in numerous ways, including its ability to be activated by ARF. Like cholera toxin, LT contains an subunit, the ADP-ribosyltransferase activity of which is latent; activation requires reduction of a single disulfide bond near the (terminus and peoteolysis between the two cysteines, releasing a catalytically active A1 protein and a smaller protein. To determine whether proteolysis plus reduction is required for appearance of the ARF allosteric site, as well as for catalytic activity, an inactive mutant of LT (LT9E112K), synthesized by Takao Tsuji, was utilized as a competitor in cholera toxin ADP-ribosyltransferase assays containing limiting amounts of ARF. In LT(E112K), glutamate-112 in the A1 protein, which is believed to be an active site residue, is replaced with lysine; the mutant protein did not exhibit ADP-ribosyltransferase activity with or without ARF. LT(E112K) required trypsinization and reduction to become a potent, concentration-dependent inhibitor of ARF stimulation of cholera toxin ADP-ribosyltransferase activity. As expected, inhibition was reversed by increasing concentrations of ARF. Reduction or trypsinization alone did not generate an inhibitory form of LT(E112K). These studies are consistent with the conclusion that the ARF site is not present in the latent toxin. Both proteolysis and reduction are required for expression of a functional ARF binding site as well as for catalytic activity.

In patients, the effects of cholera toxin presumably occur in the jejunum at body core temperature. For assays with agmatine as a model substrate (or for

auto-ADP-ribosylation), however, the optimal temperature was 20-30°C. The significantly lower activity at 37°C reflected lower initial velocity, not heat-inactivation of the toxin. Phospholipids and ARFs, presumably present in the intestine at the site of toxin action, shifted the temperature optimum, so that maximal effects of ARF were noted at physiological temperatures (~37°C) in the presence of cardiolipin, phosphatidylinositol and cholate, or dimyristoylphosphatidylcholine and cholate. In fact, ARF activation was minimal at any temperature in the absence of phospholipid or detergent. There were also similar effects on other cholera toxin-catalyzed reactions such as the hydrolysis of NAD and the ADP-ribosylation of $G_{s\alpha}$. Kahn and Gilman initially reported the importance of detergent for ARF stability and activity and had found that high affinity GTP binding by sARFII (later identified as ARF3) was observed only in the presence of dimyristoylphosphatidylcholine and cholate. These lipids similarly lowered the apparent EC_{50} for GTP in the ARF-activated cholera toxin assay. Differences in the effects of phospholipids on activities of recombinant ARFs from the three classes were later observed.

Most of the information on ARF function in cells is related to vesicular transport in the Golgi, probably involving class 1 ARFs. ARFs have also been implicated in ER to Golgi transport, endocytosis, and nuclear membrane assembly. Some of the most extensive, detailed, and successful studies of vesicular transport, including vesicle formation, targeting and fusion have involved intra-Golgi transport utilizing an *in vitro* system initially devised in Rothman's laboratory. In this system, they defined the roles of ARF and coatomer in vesicle formation, identified the NEM-sensitive fusion (NSF) protein, and worked out other requirements for vesicle fusion with target membranes. To initiate intracellular membrane vesicle formation, binding of ARF·GTP is required. In the cell, ARF·GDP is cytosolic. Interaction with a specific guanine nucleotide-exchange protein accelerates exchange of GDP for GTP, producing ARF-GTP, which can bind to phospholipids or membranes. In cells, it binds presumably to a specific membrane region at which vesicle budding will occur. Whether this involves a specific ARF "receptor" protein or some other means of defining the interaction site has not been established.

Brefeldin A (BFA), a fungal lipid metabolite, disrupts Golgi function and inhibits the binding of soluble high molecular weight protein complexes termed coatomers to Golgi fractions, which requires prior binding of ARF·GTP. Exchange of GDP bound to ARF 1 for GTP was shown by other investigators to be enhanced by Golgi membranes in a BFA-sensitive reaction, leading to the conclusion that a guanine nucleotide-exchange protein (GEP) was the BFA target. To establish the site of BFA action and to characterize the guanine nucleotide-exchange reaction, we partially purified a soluble GEP from bovine brain. GEP-dependent exchange of nucleotides on ARFs 1 and 3 was markedly enhanced by phosphatidylserine, although cardiolipin was, in fact, more effective than phosphatidylserine in enhancing ARF-stimulated cholera toxin activity. The crude GEP behaved as a molecule of ~700 kDa, whereas after partial purification, it behaved on Ultrogel AcA 54 chromatography as a protein of ~60 kDa. With purification, GEP activity became BFA-insensitive, consistent with the conclusion that the exchange protein is not itself the BFA target. It appears that the loss of BFA inhibition with purification is related to separation of a ~60-kDa GEP molecule from another protein(s) that is sensitive to BFA, with which it was initially associated in the

larger complex. That complex may include other regulatory proteins and/or proteins that interact with ARF along with coatomer to initiate budding. Obtaining GEP activity in the soluble fraction clearly facilitated attempts to purify it and thereby to find that BFA-inhibition of ARF-GEP activity depends on the presence of another protein.

3. ADP-ribosylation of Proteins in Animal Cells

We initially assumed that ADP-ribosylation reactions catalyzed by the bacterial toxins in animal cells mimicked the actions of endogenous enzymes and found an enzyme in avian erythrocytes that catalyzed the ADP-ribosylation of arginine and arginine residues in proteins. Subsequently, it was shown that a family of such NAD:arginine ADP-ribosyltransferases exists in avian tissues with differences in intracellular localization, and kinetic, physical, and regulatory properties. Similar enzymes were later identified by others in mammalian tissues. If ADP-ribosylation were to be a reversible modification of proteins, as might be expected for a regulatory process, an enzyme should exist that removes the ADP-ribose moiety, regenerating free arginine. ADP-ribosylarginine hydrolases were identified in avian tissues, and subsequently in mammalian cells. Thus, the components of an ADP-ribosylation cycle appears to exist in higher eukaryotic species with NAD:arginine ADP-ribosyltransferase catalyzing the transfer of ADP-ribose from NAD to proteins and ADP-ribosylarginine hydrolases cleaving the ADP-ribose-protein bond, releasing ADP-ribose and regenerating free (arginine) protein. Until this year, however, no substrate(s) had been identified, and thus the physiological function of these enzymes remained in question.

NAD:arginine ADP-ribosyltransferase mRNA and protein were identified in skeletal and cardiac muscle, but not in other tissues, from several mammalian species. Last year, it was noted that the deduced amino acid sequence was consistent with that of a glycosylphosphatidylinositol-anchored protein. Using a mouse muscle cell line capable of differentiating in culture into myotubes, it was found that the transferase activity, which increased during differentiation was located on the external surface of the cells. After incubation of cells with radiolabelled NAD, one major protein was ADP-ribosylated. Preliminary identification of the substrate as the α subunit of an integrin $\alpha\beta$ heterodimer (based on migration in SDS-polyacrylamide gels with and without reduction) was confirmed after its isolation by laminin affinity chromatography and microsequencing, which was consistent with $\alpha 7$.

The selective expression of ADP-ribosyltransferase and integrin $\alpha 7$ in cardiac and skeletal muscle, their parallel developmental appearance, and the apparent specificity of ADP-ribosylation are consistent with a regulatory association between these two proteins. Integrin $\alpha 7$ in association with integrin $\beta 1$ is a laminin-binding protein. ADP-ribosylation may affect interaction of muscle cells with the extracellular matrix and/or intracellular signalling by the integrin. The location of the ADP-ribosyltransferase appears to be critical for its ability to modify integrin. Release of the enzyme from the cell surface using PI-PLC significantly reduced the ADP-ribosylation of the 97-kDa integrin protein. Moreover, using membranes rather than intact cells resulted in significant ADP-ribosylation of non-integrin proteins. This difference in ADP-ribosylation pattern is consistent with the

conclusion that the ADP-ribosylation of integrin was indeed occurring in intact cells.

The rat alloantigen RT6.2, which encodes a GPI-linked protein of ~24 kDa, is expressed primarily in post-thymic lymphocytes. Its expression may be related to diabetes mellitus, although more recent findings seem inconsistent with those claims. The human and rabbit skeletal muscle NAD:arginine ADP-ribosyltransferases have significant amino acid sequence similarity to RT6.2 throughout the coding region, although the difference in sizes of the two proteins necessitated insertion of numerous gaps to achieve alignment. One stretch of conserved amino acids was located in the acidic region in the carboxy terminal one-third of the protein, which may be part of the catalytic site.

To determine whether RT6.2 has an activity similar to that of the ADP-ribosyltransferase, the RT6.2 gene was expressed in rat mammary adenocarcinoma (NMU) cells under control of a dexamethasone-sensitive promoter. RT6.2-transformed, but not vector-transformed, cells exhibited a cell surface-associated NAD glycohydroase (NADase) activity that was released by incubation with PI-PLC. The released NADase had a mobility on SDS-polyacrylamide gels consistent with a protein of 22-24 kDa, i.e., the size of RT6.2. The RT6.2 gene product did not catalyze the transfer of ADP-ribose to simple guanidino compounds, and thus differed from the transferase. In contrast to CD38, a cyclic ADP-ribose synthase, and perhaps other NADases, it neither synthesized nor degraded cyclic ADP-ribose. Thus, RT6.2 would appear to be an NADase; further, these studies suggest that the conserved residues in the catalytic site of RT6.2 NADase are similar to those found in the bacterial toxin ADP-ribosyltransferases and the mammalian muscle transferases.

In earlier studies, we had purified, microsequenced, and cloned a rat brain ADP-ribosylarginine hydrolase. No similar proteins have been identified in data bank searches. To determine regions crucial for activity, cross-species conservation of structure was evaluated by cloning rat, mouse, and human hydrolases. The mouse hydrolase was 92% identical in coding region nucleotide sequence and 94% identical in deduced amino acid sequence to that of the rat. The human enzyme was 82% identical in coding region nucleotide sequence and 83% and 82% identical to rat and mouse deduced amino acid sequences, respectively. The rat and mouse hydrolases were dithiothreitol-dependent and had five cysteines in identical positions. The human hydrolase was dithiothreitol-independent with only four of the five cysteines present in rat and mouse. Based on the hypothesis that the differences in dithiothreitol sensitivity were related to the differences in the fifth cysteine, mutant human (S103C) and rat (C108S) hydrolases were synthesized. The mutant human enzyme was dependent on dithiothreitol for activity, whereas the rat mutant hydrolase was dithiothreitol-independent, consistent with that hypothesis. Affinity-purified polyclonal antibodies against the rat hydrolase reacted with the recombinant wild-type enzyme, but only weakly with the rat C108S mutant hydrolase; they did not react with the human wild-type or human S103C mutant. These data suggest that cysteine 108 in the rat hydrolase plays a critical role in dithiothreitol dependence and is important in an epitope of the rat enzyme.

Annual Report of the Laboratory of Chemical Pharmacology
October 1, 1993 to September 30, 1994

During the past decade the Laboratory of Chemical Pharmacology has focused on various aspects of drug-induced neoantigen formation and the responses of mast cells to IgE antibodies. For example, the Section on Pharmacological Chemistry headed by Dr. Lance Pohl studies the mechanisms by which chemical reactive metabolites, such as the trifluoroacetyl halide formed from halothane, are synthesized and form neoantigens, and the Section on Cellular Pharmacology headed by Dr. Michael Beaven studies the mechanisms by which antigens cause the cascade of events that lead to the release of inflammatory mediators from mast cells. During the past few years, however, the Laboratory has expanded its interests into several other areas. The Section of Enzyme Drug Interaction headed by Dr. James Gillette has studied factors that govern the substrate specificity and the pattern of products formed by the various cytochrome P-450 enzymes. The Section on Drug Tissue Interaction headed by Dr. Gopal Krishna studies the expression of messenger RNA for tissue specific isoforms of various enzymes, such as guanylate cyclase and heat shock proteins. Dr. Ingeborg Hanbauer studies mechanisms of dopamine uptake and release. In addition, the Sections have collaborated on studies of (a) mechanisms of drug induced alterations of hemoproteins including cytochrome P-450 enzymes and (b) the biochemistry and pharmacology of nitric oxide.

Activation of Mast Cells

Tissue mast cells and blood basophils, when stimulated by antigens via receptors for IgE (FcϵR1), initiate immediate hypersensitivity reactions, through rapid discharge of secretory granules and synthesis of metabolites derived from arachidonic acid, as well as delayed inflammatory responses, through synthesis and release of a broad repertoire of cytokines. The objective of the Section on Cellular Pharmacology is the identification of the signalling pathways for each of these functional responses. It has utilized cultured rat RBL-2H3 cells which, like mast cells, contain FcϵR1 and, like mast cells and T-cells, a novel adenosine receptor (J. Biol. Chem. 265:745, 1990) since identified as the recently cloned adenosine A_3 receptor (J. Biol. Chem. 268:16887, 1993). In addition, the cells have been made to express muscarinic m1 receptors by gene transfection. All three receptors utilize distinct coupling mechanisms: the A_3 and m1 receptors recruit respectively, $G_{\alpha i\text{-}(2 \text{ or } 3)}$ and $G_{\alpha q/11}$ and, as shown by others, FcϵR1 recruits the tyrosine kinases lyn and syk. They all, however, initiate a similar array of signals namely, the activation of phospholipases C and D, mobilization of Ca^{2+} from the same intracellular pool with replenishment of this pool by similar influx mechanisms, and the activation of various kinases which include isozymes of protein kinase C, Ca^{2+}-dependent myosin light chain kinase and mitogen-activated protein (MAP) kinases. Stimulation via the A_3 receptor, however, elicits a transient activation of phospholipase C and MAP kinases but sustained activation of phospholipase D and protein kinase C. Another notable difference is that stimulation via FcϵR1 results in tyrosine phosphorylation of a wide spectrum of proteins including

phospholipase C γ1.

We have now identified the specific signals that initiate each of the functional responses by utilizing the differences noted above and two other strategies. The strategies include the use of washed permeabilized-cells, which lose all isozymes of protein kinase C but retain all of the other signalling elements, and the use of inhibitors of Ca^{2+}-mobilization (e.g. EGTA), protein kinase C (e.g. Ro31-7549), the MAP kinase cascade (e.g. quercetin) and production of Golgi-derived vesicles (e.g. brefeldin A). For example, we showed last year that the secretory response to antigen can be reconstituted in washed-permeabilized cells by provision of physiological concentrations of Ca^{2+} and certain isozymes of protein kinase C to indicate that these two elements transduced the required signals for secretion (J. Biol. Chem. 268:1749 &7372, 1993). Also, phospholipase D provides a reinforcing signal probably through the generation of diacylglycerols (from phosphatidic acid), which activate protein kinase C; agonists of the A_3 receptor have weak secretagogue activity by themselves, but they markedly synergize secretion induced by other stimulants through activation of phospholipase D.

With respect to release of arachidonic acid, we showed this year that this release was dependent exclusively on the activation of the recently cloned 83 KDa cytosolic phospholipase A_2 (cPLA$_2$) through its phosphorylation by MAP kinase. The association of cPLA$_2$ with membranes and the subsequent hydrolysis of membrane lipids, however, were dependent on modest elevations of Ca^{2+} and an unidentified diffusible factor. With respect to production of the cytokine TNFα, the activation of protein kinase C and elevation of free Ca^{2+} are necessary but insufficient because a third signal, either the activation MAP kinase or recruitment of a tyrosine kinase, is necessary for optimal production. Finally, release of newly formed TNFα occurs via Golgi and is totally dependent on protein kinase C and a rise in free Ca^{2+} (J. Immunol. in press). Thus, release of secretory granules and Golgi-derived vesicles appear to be regulated by the same signals although previosly such vesicles were thought to be secreted constitutively.

Our interest now is the elucidation of the role of G proteins in mediating late signals for granule/vesicle trafficking. Recently we showed that the classic mast cell secretagogue, compound 48/80, acted at a late step in exocytosis by directly activating $G_{\alpha i\text{-}3}$ in the plasma membrane (Science, 262:1569, 1993). We also have indications that $G_{\alpha s}$ has a similar role in antigen-stimulated cells. In summary, our two major achievements this year have been the demonstrations that trimeric G proteins have a role other than coupling of receptors to effector systems and that secretion of proteins via Golgi can be a receptor-regulated process.

Mechanisms of Drug-induced Toxicities

Neoantigens of halothane: Patients suffering from halothane ($CF_3CHClBr$) hepatitis have antibodies in their sera that are directed against distinct liver microsomal polypeptides that have been covalently modified by the reactive trifluoroacetyl chloride (CF_3COCl) metabolite of

halothane. The Section of Pharmaceutical Chemistry has been exploring the possibility that the patients' toxicity was initiated by trifluoroacetylated (TFA) liver immunogens, which led to immune reactions directed against one or more of the TFA or native microsomal antigens. This year it discovered that the formation of the TFA liver antigens occurred in a stereoselective manner. The R-isomer of halothane was metabolized *in vivo* by liver microsomal cytochromes P-450 to form approximately 2-3 fold larger amounts of TFA microsomal proteins, as determined by immunoblotting and immunohistochemical analyzes, than was the S-isomer. Since both enantiomers appeared to have the same anesthetic potency in this study, the results suggest that the S-isomer would be a safer inhalation anesthetic than either the R-isomer or racemic halothane, which is the composition of the drug used clinically. Since other inhalation anesthetics, such as enflurane, isoflurane, and desflurane are also used clinically as racemic mixtures, it is possible that their metabolism and associated hepatotoxicity may occur in a stereoselective manner. An important question that remains to be determined is how the TFA liver antigens, which are concentrated in the lumen of the endoplasmic reticulum, might come in contact with the immune system to induce immune reactions and subsequent immune-mediated toxicities. In order to help answer this question, the turnover of the antigens associated with halothane hepatotoxicity was studied in primary rat hepatocytes. The major site of degradation of the TFA antigens appeared to be the lysosomes, because ammonium chloride or leupeptin markedly inhibited the turnover of all of the TFA antigens, except that of the GRP 94 antigen. These findings are important because they indicate that the immune system may not come in contact with the intact TFA antigens when most individuals are exposed to halothane, because the TFA antigens are extensively degraded in the lysosomes. Perhaps patients who develop halothane hepatitis have a defect in the way they process the TFA antigens. The molecular biology of the 58 kDa antigen is being studied in order to learn more about the basis of its antigenicity and physiological function. A 1.8 kb full length clone was isolated from a human liver cDNA library with the use of anti-58 kDa sera. The sequence of the human protein showed 92% and 90% homology to that of the proteins encoded by mouse and rat liver cDNAs, respectively. The human cDNA has been expressed in COS-7 and is currently being expressed in Sf-9 cells in order obtain enough of the protein for further studies.

Nonsteroidal anti-inflammatory drugs (NSAIDs): Last year an antibody was developed that recognized tissue proteins that contain covalently bound diclofenac, the most widely used NSAIDs. This year the diclofenac adducts have been further characterized in livers of rats. Subcellular fractionation of liver homogenates from diclofenac-treated rats showed that a 50 kDa microsomal protein and 110, 140, and 200 kDa plasma membrane proteins were labeled preferentially. Immunofluorescence studies of isolated hepatocytes and immunohistochemical analysis of liver slices from diclofenac-treated mice and rats confirmed that plasma membrane proteins were labeled by diclofenac metabolites and showed that the bile canalicular domain of the plasma membrane was a major site of diclofenac adduct formation. These results demonstrate that plasma membrane proteins are major targets of diclofenac metabolites in vivo, and the presence of these adducts may be important in the mechanism of diclofenac hepatotoxicity.

Mechanisms of Metabolism-based Alterations of Hemoproteins

Although many chemically reactive metabolites are sufficiently long lived to escape the enzymes that catalyze their formation and to become covalently bound to many other proteins and other

macromolecules, some chemically reactive metabolites are short lived and never leave the active site of the enzyme. These short lived metabolites may inactivate hemoproteins by 1) reacting with the heme, 2) reacting with amino acid groups in the active site of the heme enzyme, or 3) reacting with either the protein or the heme to form radicals that lead to the covalent binding of the heme to the protein. Occasionally, a "long-lived chemically reactive metabolite" may also inactivate an enzyme by entering the active site of the enzyme for which it has high affinity and reacting with groups within it. During the past few years, the laboratory has discovered systems illustrating each of these mechanisms of inactivation.

Horseradish peroxidase: The porphyrinogenic agent, 3,5-dicarboxy-3,6-dimethyl-4-ethyl-1,4-dihydropyridine (DDEP), destroys various cytochrome P-450 isozymes by different mechanisms. Cytochromes P-450 2C11 and 2C6 appear to be inhibited through ethylation of the heme prosthetic groups, whereas inhibition of cytochrome P-450 3A occurs through covalent binding of the heme to the apoprotein. Since adequate amounts of these isoforms of cytochrome P-450 are not available, Dr. Sugiyama has studied the oxidation of DDEP by $K_3Fe(CN)_6$ and by a combination of horseradish peroxidase and H_2O_2. Both systems form 3,5-dicarboxy-3,6-dimethyl-4-ethyl-pyridine (EDP) and 3,5-dicarboxy-3,6-dimethyl-pyridine (DP), but the relative amounts depend on the pH of the medium; under neutral and acetic conditions EDP predominated, whereas under basic conditions DP predominated. Moreover, under basic, but not acidic conditions, horseradish peroxidase was inactivated and the heme group was altered. A combination of mass spectroscopic analysis and NMR indicated that the altered heme contained a 2-hydroxyethyl group attached to the δ-meso carbon of the heme. When similar experiments were performed with ethylhydrazine as the potential ethyl group donor, the heme of the peroxidase was converted to both δ-meso-ethyl heme and the δ-meso-1-hydroxyethyl heme. The origin of the oxygen remains in doubt, but it cannot be hydrogen peroxide.

Myoglobin: Dr. Osawa has previously shown that oxidative damage to heart myoglobin leads to heme alteration and transformation of the protein to an oxidase. Intracellular loading of the oxidatively modified protein was found to cause cell death. Furthermore, oxidative modification of the hemoprotein prostaglandin H synthase, a process that is thought to be involved in regulation of arachidonic acid metabolism, results in prosthetic heme alterations similar to that of myoglobin. We have further characterized these reactions in the hopes of identifying molecular markers for oxidative damage. We have found that a novel heme adduct with a mass of 632, consistent with an oxyheme derivative is formed from myoglobin and prostaglandin H synthase. Although previous reports have indicated that oxidatively modified heme leads to a mesohydroxylated adduct, which is widely accepted as an intermediate in the "normal" course of heme metabolism by heme oxygenase, the product we have isolated is not this adduct as judged by proton NMR. The exact structure is currently being investigated in the hopes of developing an assay for monitoring in vivo oxidative damage to hemoproteins. Experiments with ^{18}O forms of water and hydrogen peroxide revealed that oxygen in the product was derived from water. NMR studies revealed that the products probably are 18-hydroxy-17-spirolactone-heme and 17,18-dihydroxy-heme. The mechanism of oxidation by peroxide thus clearly differs from the mechanism of heme-oxygenase.

Synthesis and Actions of Nitric Oxide (NO)

Dopamine release: Dr. Hanbauer has reported that, in slices of striatum from rat brain, NO mediates the release of dopamine from presynaptic neurons evoked by N-methyl-D-aspartate, a postsynaptic glutamate receptor agonist. But the mechanism of the NO-mediated release of dopamine remains obscure. Many possibilities have been eliminated. It is not mediated either by activation of guanylate cyclase, or by increases in intracellular calcium. Moreover, NO causes release under anaerobic conditions, which precludes the possibility that it is caused by oxidized products of NO or oxygen radicals. Furthermore, the release cannot be due to inhibition the uptake of dopamine, because NO does not affect dopamine uptake into neurons.

NO effects on the toxic effects of superoxide and hydrogen peroxide. Dr. Hanbauer and her collaborators have shown that NO inhibits the toxic effects of superoxide and hydrogen peroxide generated by hypoxanthine and xanthine oxidase on fetal mesencephalic neurons. Moreover, NO by itself is not toxic to those neurons. Although the mechanism of the protective effects is obscure, it may be relevant that NO facilitates the single electron oxidation of dopamine and ascorbic acid, which suggests that the toxicity of superoxide and hydrogen peroxide does not depend on dopamine oxidation. In other experiments Dr. Hanbauer has found that NO decreases the formation of hydroxyl radicals by ferric-citrate complexes as measured by the hydroxylation of salicylic acid.

NO synthesis in human retina. Using reverse transcriptase polymerase chain reaction techniques, Dr. Krishna and his associates have shown that the NO synthase expressed in human retina is probably the same as that expressed in human brain.

Expression and inhibition of rat brain nitric oxide synthase. Dr. Krishna and his associates have expressed rat brain NO synthase in CHO cells and have shown that the enzyme can be inhibited by conavanine, an arginine analogue, present in Jack Beans, by a mixed mechanism. Conavanine, however, was not as potent an inhibitor as nitro arginine or N-methyl arginine.

Irreversible inactivation of NOS: Last year Dr. Osawa reported that phencyclidine, a psychotomimetic and drug of abuse, irreversibly inactivates brain NOS *in vitro*. This was an important finding because it indicated that NOS could metabolize compounds, other than its natural substrate arginine, into reactive metabolites, which might inactivate NOS. This mechanism may be responsible, at least in part, for the central nervous systems effects associated with phencyclidine abuse. This year these findings have been extrapolated to the study of some antihypertensive agents. It was found that these agents irreversibly inactivated neuronal NOS *in vitro* and *in vivo* at pharmacologically relevant doses. This discovery may help explain how many antihypertensive agents cause impotency. This year neuronal NOS was expressed in Sf9 cells in order to obtain enough of the enzyme to study how drugs inactivate it. It has been postulated that nitric oxide (NO) can react with tyrosine residues of proteins to form 3-nitrotyrosine derivatives. This covalent alteration of proteins could have a physiological function or possibly be involved in pathological conditions produced by excessive NO. In order to better understand the biological role of this covalent modification of proteins, this year a polyclonal antibody was developed that can recognize proteins containing 3-nitrotyrosine residues.

Neurotoxicity in dopamine neurons

Dr. Hanbauer and her collaborators have found that, in primary cultures of mesencephalic neurons, stimulation of non-NMDA type receptors destabilizes calcium homeostatis earlier in dopaminergic neurons than in other neurons. When both kinds of cells are exposed to high concentrations (50 μM) of AMPA, glutamate, kainate or quisqualate for 1-5 minutes (which causes 3-5 fold increases in the intracellular concentrations of calcium), followed by superfusion of the cells with drug-free medium, the concentration of calcium returned to normal in the nondopaminergic cells but not in the dopaminergic cells. The dopaminergic cells, but not the nondopaminergic cells, died 6-7 hours later.

Mechanisms of Cytochrome P-450 Enzymes

Last year the Section of Drug Enzyme Interaction developed procedures, based on the effects of deuterium substitution on the rate of formation of the various metabolites, that permit us to determine the mechanism by which various cytochrome P-450 systems convert a substrate to several metabolites. Using these procedures we have developed evidence indicating that cytochrome P-450 2C11 forms A-ring and D-ring metabolites of testosterone by the dissociative mechanism in which the (EOS) complexes dissociate and than reassociate in different orientations. During these studies we found that cytochrome P-450 2C11 formed a second generation metabolite, 16α-hydroxyandrostenedione (16-OHA), as well as 16α-hydroxytestosterone (16-OHT) and androstenedione (A) even at the highest concentrations of testosterone used in the experiments. But it wasn't clear whether the (16-OHA) was formed directly from the enzyme-metabolite in complex or indirectly by dissociation of the complex, and reformation of the complex. To elucidate the mechanism we developed mathematical approaches to determine the relative sizes of the rate constant for the dissociation of the enzyme-metabolite complex and the rate constant for the activation of the enzyme-metabolite leading to the formation of (16-OHA). With this approach, we determined that (16-OHA) is formed solely from (A) and that the rate constant for dissociation of the (E-A) complex cannot be more than 7 times the rate constant for activation of the complex.

Retinal guanylate cyclase

For many years the Section on Drug Tissue Interaction has been studying possible etiologies of retinitis pigmentosa. Toward this end, Dr. Krishna and his associates have studied the expression of the messenger RNAs for the guanylate cyclases and other proteins in human retinas. Using the reverse transcriptase - polymerase chain reaction approach, they have found that messenger RNAs for the guanylate cyclases, named atrial naturetic peptide receptors types A and B, present in human brain, are also present in human retinas. In addition they have found that human heat shock factor 1, which governs the synthesis of heat shock proteins, is also present in human retinas.

Annual Report
Laboratory of Molecular Cardiology
National Heart, Lung, and Blood Institute
October 1, 1993 through September 30, 1994

The Laboratory of Molecular Cardiology investigates the regulation, expression and function of contractile proteins and homeodomain proteins. We are particularly interested in the mechanisms responsible for regulating the contractile proteins in smooth muscle and nonmuscle cells as well as the factors that govern the expression of the genes encoding the contractile proteins. In addition, we are studying a particular set of homeobox genes that play a role in the early development of Drosophila and mammalian embryos. Our purpose is to understand the mechanism of action and regulation of a number of genes involved in cell differentiation, development, and motility. By studying the genes, mRNA, and proteins involved in these developmental and contractile processes, we hope to understand the mechanisms by which cells differentiate, alter their phenotype, migrate, change their shape, move their membrane receptors, secrete cellular products, and proliferate. We plan to use this information to understand both normal and disease processes.

Below is a summary of the various projects under study during October 1993 to September 1994. The following findings were of particular note: 1) The first intron of the gene encoding human nonmuscle myosin heavy chain-A was found to contain a number of regions possessing cell type-dependent transcriptional enhancer activities. 2) A minigene of myosin heavy chain-B was constructed that expresses mRNA capable of undergoing alternative splicing to produce a neuronal-specific myosin isoform. 3) The homeobox gene *NK-4* was found to be a direct target of *twist*, which is a mesoderm determinant in Drosophila embryos. 4) Using the yeast two-hybrid system, four novel genes encoding proteins that could interact with the *NK-1* homeodomain transcription factor were identified. 5) New point mutations in the myosin heavy chain from patients with hypertrophic cardiomyopathy were analyzed using the *in vitro* motility assay and found to be abnormal. The myosin heavy chain mutations were mapped onto the crystal structure of chicken skeletal muscle myosin subfragment-1. 6) A neuronal-specific isoform of myosin heavy chain-B was found to be phosphorylated at a specific site by proline-directed kinases *in vitro*. 7) Xenopus myosin heavy chain-B was found to be phosphorylated on serine-214 during progesterone-induced meiosis of oocytes, when cdc2 kinase is active, but not during interphase, when it is inactive.

Growth and Differentiation of Smooth Muscle and Nonmuscle Cells (S. Kawamoto, Z01 HL 01665-19 MC). The purpose of the present studies is to identify the various elements that are involved in upregulating nonmuscle myosin heavy chain (MHC) gene expression in C2 myoblasts and fibroblasts and to identify the regulatory elements downregulating nonmuscle myosin heavy chain gene expression in myotubes. Recently, two fragments from the first intron of the MHC-A gene, 0.5 kb and 2.8 kb in size, were found to cause a 3~10-fold increase in transcriptional

activity in NIH 3T3 fibroblasts and C2 myoblasts, but not in differentiated C2 myotubes. In a separate study, in order to identify the mechanism underlying neuron-specific alternative splicing of MHC-B, human genomic clones, which encode this region, have been cloned. In an effort to localize the critical region of pre-mRNA responsible for regulated alternative splicing, a minigene with deletions and/or mutations in the appropriate area has been constructed using an eukaryotic expression vector. Analysis of the mRNAs derived from the minigene following transfection into various cultured cells suggests that it is capable of undergoing splicing to produce a neuronal-specific myosin isoform.

Myosin and Caldesmon Phosphorylation in Nonmuscle Cells (G. Bazile, J.R. Sellers, Z01 HL 01785-15 MC). Previously, it has been shown that caldesmon is phosphorylated when human platelets are treated with prostacyclin, at sites phosphorylated *in vitro* by cAMP-dependent protein kinase. The purpose of this study is to determine the exact location of the sites phosphorylated by cAMP-dependent protein kinase. Using a recombinant caldesmon fragment purified from *E. coli*, one of these sites was localized to the N-terminus and was found to be serine 202. Preliminary results using intact purified platelet caldesmon have confirmed the above observation. In addition, a second site of phosphorylation has been tentatively identified as being serine 263. Presently, a number of different techniques, including cyanogen bromide cleavage and PVDF membrane electroblotting of peptides, are being employed to identify the sites of phosphorylation.

Role of Phosphorylation as a Regulatory Mechanism in Muscle Contraction (J.R. Sellers, Z01 HL 01786-15 MC). Kinase related protein (KRP), also referred to as "telokin", represents the carboxy-terminal 20,000 kD of myosin light chain kinase. The expression of KRP is controlled by its promoter which lies within an intron in the myosin light chain kinase gene. The purpose of this project is to understand the function of KRP. Work performed in this laboratory has shown that KRP binds to unphosphorylated smooth muscle and nonmuscle myosin and may play a role in stabilizing myosin filaments. Unphosphorylated vertebrate smooth muscle and nonmuscle myosin filaments are depolymerized by the addition of ATP at physiological ionic strength. The solubilized myosin adopts a folded monomeric conformation with a sedimentation coefficient of 10S. KRP prevents this depolymerization by binding to myosin, probably close to the "neck" region, i.e., the junction of the head and the subfragment-2 region of the myosin heavy chain. One mol of KRP appears to bind per mol of myosin. Thus, it is proposed that KRP functions to stabilize myosin filaments, possibly by binding to the same sequence of amino acids in the neck region of myosin to which the tail part of the myosin molecule binds in forming the 10S conformation.

Neuronal Cell-specific Expression of Vertebrate Nonmuscle Myosin Heavy Chains (K. Itoh, R.S. Adelstein, Z01 HL 04208-08 MC). Previous work has demonstrated that there are unique isoforms of nonmuscle myosin heavy chain-B that are expressed in chicken and mammalian (including human) neuronal cells. These isoforms, which are generated by alternative splicing of pre-mRNA, differ from the myosin heavy chain-B isoform present in a large number of nonmuscle cells in that

they contain inserted cassettes of amino acids near the ATP binding region and/or near the actin binding region. There is a marked species difference between the distribution of the inserted isoforms in adult tissue. Whereas the 10 amino acid insert near the ATP binding region is poorly expressed in chicken brain, it makes up most of the myosin heavy chain-B mRNA expressed in the human cerebrum and approximately 90% of the myosin heavy chain-B mRNA in the human retina. The 21 amino acid insert is abundantly expressed in the chicken cerebellum and human cerebrum, but is absent from human retina and human cell lines. Using a variety of neuronal-derived cell lines, we studied conditions that increased the expression of the 10 amino acid insert present near the ATP binding region. Employing human retinoblastoma and neuroblastoma cell lines, we are able to increase the expression of the inserted isoform using a number of agonists or using serum deprivation. In each case, expression of the inserted isoform correlates with cell differentiation and inhibition of cell division.

Myosin Phosphorylation and the Regulation of Contractile Activity (C.A. Kelley, R.S. Adelstein, Z01 HL 04210-07 MC). Previously, an isoform of vertebrate nonmuscle myosin heavy chain-B with an inserted sequence of 10 or 16 amino acids (Takahashi et al., *J. Biol. Chem.* **267**: 17864, 1992; Bhatia-Dey et al., *Proc. Natl. Acad. Sci. USA* **90**: 2856, 1993) and isoforms of smooth muscle myosin heavy chains, with an insertion of 7 amino acids (Kelley et al., *J. Biol. Chem.* **268**: 12848, 1993) at a site near the ATP binding region in the myosin head, have been reported. In chicken smooth muscle, the insert occurs in intestinal, but not in vascular myosin. The presence of the insert correlates with a faster velocity of movement of actin filaments in an *in vitro* motility assay and a higher actin-activated MgATPase activity. Preliminary evidence obtained in collaboration with J.R. Sellers suggests that the insert increases the rate of ADP release, thereby increasing enzymatic activity. In cultured Xenopus XTC cells, we have identified two inserted myosin heavy chain-B isoforms as well as a noninserted myosin heavy chain-A isoform by immunoblotting of cell extracts. When myosin was immunoprecipitated from XTC cells and phosphorylated with cdc2 kinase, a single serine residue with a -ser-pro-lys- amino acid sequence was the only site phosphorylated. Moreover, the same serine site was phosphorylated during maturation of Xenopus oocytes when the cdc2 kinase-containing maturation promoting factor is activated, but not in G2 interphase-arrested oocytes. These results demonstrate that myosin heavy chain-B phosphorylation by cdc2 kinase is tightly regulated during the meiotic cell cycle.

Expression of Mutant Vertebrate Myosin I's (F. Wang, J.R. Sellers, Z01 HL 04213-05 MC). We are using the baculovirus/Sf9 insect cell system, in collaboration with M.A. Conti, to express full-length chicken brush border myosin I heavy chains along with calmodulin as well as a truncated myosin head fragment. An interesting difference between vertebrate myosin I's and myosin I from lower eukaryotes such as Acanthamoeba and Dictyostelium relates to the mechanism of the regulation of their activity. Whereas myosin I's from lower eukaryotes require phosphorylation of a serine located in the myosin head domain for activity, vertebrate myosin I's are constitutively active. Sequence alignments of vertebrate and Acanthamoeba myosin

I's reveal that the vertebrate proteins have a negatively charged amino acid at the position where the Acanthamoeba protein has a phosphorylatable serine. It is our purpose to explore, using site-directed mutagenesis along with the baculovirus/Sf9 system, whether a negative charge at this site is essential for the actin-activated MgATPase activity and for the movement of actin filaments over myosin heads in the *in vitro* motility assay.

Structure and Function of Cardiac Myosin (G. Cuda, C. Sable, J.R. Sellers, Z01 HL 04217-04 MC). Hypertrophic cardiomyopathy (HCM) is an autosomal dominant inherited disease representing an important cause of sudden cardiac death in an otherwise healthy young individual. The phenotype has been linked in some kindreds to the β-myosin heavy chain gene, localized on the long arm of chromosome 14. Over thirty mutations resulting in substitution of single amino acids have been described. These mutations are localized in the head and head-rod junction regions of the β-myosin heavy chain. We are studying the functional consequences of these mutations on the enzymatic activity of β-myosin using an *in vitro* motility assay in which fluorescently labeled actin filaments are moved by the wild-type and mutant myosins which are bound to a glass surface. We have examined the β-myosin from slow skeletal and cardiac muscle of patients with HCM linked to a number of point mutations in the β-myosin heavy chain gene. The results demonstrate that some of the β-myosin heavy chain mutations in patients with HCM are associated with an abnormal actomyosin interaction which may be the basis of their disease.

Expression of Nonmuscle Myosin Isoforms in Eukaryotic Cells (M.A. Conti, R.S. Adelstein, Z01 HL 04218-04 MC). All eukaryotic cells contain one or more isozymes of the nonmuscle myosin II heavy chain. Two forms of the nonmuscle myosin heavy chain have been identified and have been found to be the products of two different genes. One of the isozymes, myosin heavy chain-B, is also subject to alternative splicing of messenger RNA to yield four possible variants. We would like to understand the function of the two myosin heavy chain isoforms as well as the function of the insertions in the head region of the myosin heavy chain-B molecule. To do this, we plan to over-express the head and rod fragments of human and frog myosins (Xenopus laevis) in cell lines of the respective species. In order to have genes for both myosin heavy chain-B and myosin heavy chain-A, we have recently cloned the cDNA encoding myosin heavy chain-A isoforms in the Xenopus laevis. These clones are presently being sequenced. The rod and head clones, along with those for the myosin heavy chain-B inserted isoforms, will be used for transfection into the frog cell lines, A6 and XTC.

Expression and Site-directed Mutagenesis of Nonmuscle Myosin Heavy Chains (R.S. Adelstein, J.R. Sellers, Z01 HL 04219-04 MC). We have used the baculovirus expression system to produce a heavy meromyosin-like fragment that contains a truncated form of the 200 kD chicken nonmuscle myosin heavy chain-B. Coexpression of the truncated myosin heavy chain-B, along with the 17 kD and 20 kD myosin light chains, resulted in an HMM_{exp} that was soluble at low ionic strength, was bound to rabbit skeletal muscle actin in an ATP-dependent manner, was capable

of moving actin filaments in an *in vitro* motility assay and manifested an actin-activated MgATPase activity provided that 20 kD light chain was previously phosphorylated by myosin light chain kinase. In order to study the differences in activity between the inserted and noninserted isoforms, we introduced the necessary nucleotides encoding a 10 amino acid insert near the ATP binding region into the cDNA encoding the myosin heavy chain-B and expressed this construct in the baculovirus system. Following purification of both the expressed inserted and noninserted myosin heavy chain-B isoforms, we have been able to demonstrate that the former, but not the latter, was capable of serving as a substrate for proline-directed kinases *in vitro*.

Function of Drosophila NK-homeobox Genes in Mesodermal Cell Differentiation (Y. Kim, Y.M. Lee, K.W. Chung, T.K. Park, Z01 HL 04221-03 MC). Previous genetic studies and the expression pattern of the homeobox genes, *NK-4* and *NK-3*, together with those of the *twist* gene during embryogenesis, suggested that these genes may regulate each other, and thereby control mesodermal cell differentiation. In order to understand the mechanism underlying dorsoventral development of the Drosophila embryo, which clearly may have implications for development in other species, we investigated the transcriptional control of these genes. CAT (chloramphenicol acetyltransferase) activities in CV1 cells cotransfected with CAT reporter plasmids containing various 5' upstream regions of the *NK-4* promoter along with the *twist* expression vector and the DNaseI footprinting assay defined an E1 cluster that is responsible for direct activation of *NK-4* by *twist*. These studies, taken together with previous results with *NK-3* and *NK-4*, demonstrate that *NK-4* is a direct target of *twist* which is a major determinant of mesoderm formation in the Drosophila embryo and, moreover, that the *NK-4* protein, in turn, autoactivates the *NK-4* gene itself and, finally, upregulates the *NK-3* gene, suggesting that a cascade of these gene regulators may play an important role in mesodermal cell specification during embryogenesis.

Cloning and Characterization of Myosin-related cDNAs from Xenopus Laevis (N. Bhatia-Dey, R.S. Adelstein, Z01 HL 04222-03 MC). In collaboration with Dr. Milan Jamrich (FDA), we have cloned a cDNA encoding a novel class of forkhead genes from Xenopus. *Xfkh3* encodes a 483 amino acid protein with a highly conserved putative DNA binding domain of 77 amino acids. Whole mount *in situ* hybridization revealed that, at stage 10.25 of the Xenopus, the transcript is expressed throughout the mesoderm. As gastrulation proceeds, the transcript becomes more restricted. In stages 11.5-12 embryos, the transcript is localized in the presomitic mesoderm and no expression is detected in the notochord and ventral mesoderm. In separate studies using whole mount *in situ* hybridization, the expression of the mRNA encoding the nonmuscle myosin heavy-B isoform, was studied during Xenopus development. *In situ* hybridization revealed that the transcript is expressed ubiquitously in blastula and gastrula stage embryos, but begins to localize in the anterior part of the embryo in late neurula. In swimming tadpoles, a strong signal is detected in differentiated somites, the eye and the branchial arches. Currently, the role of this myosin isoform is being studied during somitogenesis.

Null Mutations of Vertebrate Nonmuscle Myosin Heavy Chains (A.N. Tullio, R.S. Adelstein, Z01 HL 04223-02 MC, in collaboration with D. Accili, NIDDK). The purpose of these studies is to eliminate the genes encoding myosin heavy chain-B, myosin heavy chain-A and specific exons of myosin heavy chain-B, using the technique of homologous recombination with embryonic stem cells which are then transferred into transgenic mice. Using a genomic clone from myosin heavy chain-B, we have inserted a DNA fragment encoding the neomycin gene in the second exon replacing part of the translational start site. These mutated constructs have been transfected into mouse embryonic stem cells and are presently being screened. At present, we believe we have identified two such cell lines showing homologous recombination. These embryonic stem cells will be used to generate transgenic mice.

Function of *NK-1* Homeobox Gene in Neuro-muscular Synaptogenesis (Y. Kim, T.K. Park, K.W. Chung, Y.M. Lee, Z01 HL 04224-02 MC). One way to understand the function of the *NK-1* homeodomain transcription factor during development is to identify target genes which are regulated by *NK-1*. For this purpose, we screened for proteins which could interact with *NK-1*, using the yeast two-hybrid system which is a genetic-based method to detect protein-protein interactions. From the screening of a Drosophila adult match-maker cDNA expression library, we identified eight positive clones that showed both growth on His-deficient media and β-galactosidase activities. DNA sequence analysis revealed that these clones could be classified into five groups of novel genes. Using positive clones and various bait constructs containing different coding regions of the *NK-1* gene, we found that the middle region of the *NK-1* protein (amino acids 150-419) was involved in protein-protein interaction. It is of note that a prd-repeat (alternating His-Gln or His-Pro residues), that is also found in other homeodomain transcription factors and whose function is still unknown, is located within this region. Characterization of these clones is underway.

Regulatory Mechanisms for Nonmuscle Myosin Heavy Chain Gene Expression (K. Abe, S. Kawamoto, Z01 HL 04225-02 MC). To understand the mechanisms responsible for regulating the expression of nonmuscle myosin heavy chain genes, we have isolated genomic clones which contain the promoter and flanking region for the human nonmuscle myosin heavy chain-B gene by screening human genomic DNA libraries. Five overlapping clones were isolated and partially characterized by restriction mapping and sequencing. The clones spanned approximately 35 kb of genomic DNA and include the first and second exons. Results of primer extension studies suggest that there are multiple transcription start sites. The first exon consists entirely of untranslated sequence and the second exon contains 31 nucleotides of untranslated sequence followed by 345 nucleotides of coding sequence which includes the initiator ATG codon. Sequence analysis of approximately 1 kb upstream of the first exon showed that there is no TATA box and that the GC content is high around the putative transcription start site. Identification and characterization of the promoter region of this gene is now in progress.

ANNUAL REPORT OF THE
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1993 to September 30, 1994

Our continuing goal is to analyze the function of the kidney as a basis for understanding its pathophysiology and treating its disorders. Since the formation of urine depends upon the transport of water and solutes by kidney tubules, understanding renal function requires analysis of these cellular processes and of their integration in the kidney. Therefore, we are studying transport by cells in general and kidney cells in particular, as well as the mechanisms, hormonal and other, that support and control transport and metabolism.

Studies in single renal tubules

Studies by Nielsen, Chou, Kishore, and Knepper, along with D. Marples and E.I. Christensen of Aarhus University (Denmark), have demonstrated in isolated perfused renal collecting ducts that exposure to vasopressin causes a marked redistribution of water channels from intracellular vesicles to the apical plasma membrane, thus accounting for the vasopressin-induced increase in collecting duct permeability. In these studies water permeability was measured in isolated collecting ducts and tubules were fixed for microscopy either before or after vasopressin exposure, or after vasopressin withdrawal. A special cryosubstitution procedure was developed to allow efficient labelling of the collecting duct water channels (AQP-CD) with an anti-peptide antibody. These studies provide a direct verification of the so-called "shuttle hypothesis", i.e. regulation of plasma membrane water permeability by regulated exocytosis of vesicles containing water channels.

Studies by DiGiovanni, Nielsen, Christensen, and Knepper have demonstrated that long-term elevation of circulating vasopressin levels is associated with a marked increase in water channel expression in the renal collecting ducts. Previous studies showed that restriction of water intake in the rats was associated with a marked elevation of water permeability and expression of the AQP-CD water channel in the the collecting duct. The current studies involved 5-day infusions of vasopressin via osmotic minipumps in Brattleboro rats, i.e. rats with an absence of circulating vasopressin. An approximately three-fold increase in water channel expression was seen by immunoblotting and this correlated with an approximately three-fold increase in vasopressin-stimulated water permeability in isolated perfused collecting ducts from the rats. These studies add to mounting evidence that collecting duct water permeability is regulated by vasopressin both by short-term and long-term mechanisms.

Maeda and Knepper, along with B. Smith and P. Agre (Johns Hopkins University), developed a new method to quantify expression of specific proteins in micro-dissected tubules and employed it for the measurement of levels of the

Aquaporin-CHIP (AQP-CHIP) water channel in rat renal tubules. Among all renal tubule segments, AQP-CHIP was detected only in proximal tubule segments and in segments of the thin descending limb of Henle's loop. Based on the unit permeability of AQP-CHIP measured in purification-reconstitution studies, the measured levels in the proximal tubule were sufficient to account for the water permeability coefficients measured in isolated perfused proximal tubules and thin descending limbs of Henle's loops.

Chou, DiGiovanni, and Knepper have investigated the role of oxytocin in regulation of osmotic water permeability of the inner medullary collecting duct, demonstrating that oxytocin can function physiologically as an antidiuretic hormone. Dose-response studies in isolated perfused collecting ducts demonstrated that oxytocin increases water permeability at concentrations often found in the peripheral blood, e.g. during restriction of water intake, hemorrhage, or pharmacological induction of labor with synthetic oxytocin. The hydro-osmotic action of oxytocin was blocked by antagonists of the type 2 vasopressin receptor (V2) but not by oxytocin receptor antagonists, indicating that oxytocin acts via the V2 receptor.

Studies by Kishore and Knepper have investigated the possible role of nucleotide receptors (P2u purinergic receptors) in the regulation of water permeability of the rat inner medullary collecting duct. Ten μM ATP, UTP, or ATP-gamma-S (but not ADP) added to the peritubular bath significantly and reversibly inhibited vasopressin-stimulated water permeability. In contrast, ATP did not inhibit osmotic water permeability stimulated by cyclic AMP suggesting that the action of ATP was at a step proximal to cyclic AMP action. Consistent with this view, 10 μm ATP significantly inhibited vasopressin-stimulated cyclic AMP production in inner medullary collecting duct cell suspensions. The effect of ATP to inhibit water permeability was blocked by a protein kinase C inhibitor (calphostin C). These studies support the view that ATP decreases water permeability through a protein kinase C mediated decrease in cyclic AMP production.

Transport in model epithelia

The regulation of ionic pathways by a variety of arachidonic acid metabolites was studied by Kersting and Spring. They characterized the changes in ion transport caused by inhibition of the production of arachidonic acid metabolites in cultured human pancreatic cells with and without the cystic fibrosis genetic defect. They were able to rectify the defect by treatment of the cells with inhibitors of the production of epoxygenase metabolites of arachidonic acid. These studies indicate an important role for arachidonic metabolites in the control of ion transport involved in cystic fibrosis. The arachidonic acid metabolic pathways were also shown to influence transepithelial fluid transport in a model epithelium, Necturus gallbladder, but not to affect cell volume regulation.

Chatton, Persson, Nitschke, and Spring have developed the optical microscopy instrumentation and methods to study the composition of the fluid filling the

intercellular spaces between epithelial cells. They utilize cultured renal cells (MDCK as well as LLC-PK_1), grown on glass coverslips or permeable supports to measure the pH, Na, and Cl of the spaces between the cells and to determine the diffusion coefficient of fluorescent dyes across the tight junctions between the cells as well as within the intercellular spaces.

Napathorn and Spring have confirmed and extended previous studies on the mechanism of sorbitol release from cultured rabbit papillary renal epithelial cells. They showed that the efflux of sorbitol can be inhibited by specific proteases and transport inhibitors. They synthesized several analogues of sorbitol and determined their rate of transport under both isotonic and hypotonic conditions. These studies have yielded considerable information on the selectivity of the sorbitol transporter.

Organic osmolytes

Bacterial, plant, and invertebrate animal cells are known to accumulate compatible, osmotically active, organic intracellular solutes when their environment becomes hyperosmotic. These organic "osmolytes" help maintain the intracellular milieu because they do not perturb vital intracellular macromolecules, in contrast to sodium and potassium salts which in abnormally high concentrations do perturb macromolecules. Most mammalian body fluids are not normally hyperosmotic and the cells exposed to them do not normally express organic osmolytes. The exception is the renal inner medulla in which the interstitial fluid is hyperosmotic to a variable extent because of the renal concentrating mechanism. We identified large and variable amounts of organic osmolytes in rat and rabbit inner medullary cells, namely sorbitol, inositol, glycerophosphorylcholine (GPC), taurine, and betaine.

Control of the cellular accumulation of these osmolytes is most readily studied in tissue culture. We screened several renal cell lines in hyperosmotic media and found that cells that survived accumulated the same organic osmolytes previously found in intact renal medullas. These cell lines are now being studied in detail. The findings with regard to osmotic regulation of the individual organic osmolytes are, as follows:

1. Sorbitol accumulates in GRB-PAP1 cells by synthesis from glucose, catalyzed by aldose reductase. Hypertonicity increases aldose reductase gene transcription, mRNA abundance, and protein abundance. We have cloned the rabbit aldose reductase gene and are testing the 5' flanking region in a transient expression system to determine the molecular mechanism by which hypertonicity stimulates transcription of this gene.

2. Inositol accumulates in MDCK cells when medium osmolality is increased. The mechanism is increased transport into the cells from the medium. We cloned the cDNA for the renal cell inositol transporter by expression in toad oocytes. Using the cDNA as a probe, we find increased expression of the inositol transporter in

MDCK cells exposed to hypertonic medium. Current studies are investigating the effect of accumulation of inositol and the other osmolytes on expression of the inositol transporter.

3. Betaine, like inositol, is accumulated by MDCK cells in hyperosmotic medium because of increased transport into the cells. We cloned the cDNA for the renal cell betaine transporter by expression in toad oocytes. Using the cDNA as a probe, we find increased expression of the betaine transporter in MDCK cells exposed to hypertonic medium. The cloned betaine transporter has a nucleotide sequence similar to several recently cloned rat brain transporters which constitute a new transporter family. Current studies are investigating the effect of accumulation of betaine and the other osmolytes on expression of the betaine transporter. Also, we have cloned the mouse renal medullary betaine transporter cDNA and are establishing where it is expressed relative to the GABA/betaine transporters originally identified in brain.

4. GPC. In contrast to the other osmolytes, GPC accumulation is triggered by high urea, as well as by high NaCl. Accumulation of GPC by MDCK cells in hyperosmotic medium is due to increased net synthesis. Choline is an essential precursor. The choline is incorporated into phosphatidylcholine, which is hydrolyzed to form GPC. Activities of the enzymes involved in GPC synthesis (phospholipase A_2) and degradation (GPC: choline diesterase) are being measured. Depending on the conditions, the phospholipase may be activated and/or the diesterase may be inhibited to increase GPC; effects of urea and NaCl differ. NaCl and urea added together inhibit GPC:choline diesterase. We have purified GPC: choline diesterase, and derived some of its amino acid sequence. Present work is aimed at cDNA cloning of this enzyme and preparation of antibodies against it as steps toward determining how hyperosmolality controls its activity.

5. Identification of other genes that respond to osmotic stress. In addition to the genes involved in accumulation of organic osmolytes, hypertonicity is known to increase the expression of early response genes and genes coding for heat shock proteins. There presumably are other genes that are also important for osmotic regulation that are as yet unknown. In order to find them we are employing the technique of differential display to identify mRNAs whose abundance is greater in MDCK cells exposed to high NaCl. Several different cDNAs have been cloned that are induced by hypertonicity. At present we are sequencing and characterizing them.

Accumulation of organic osmolytes in response to osmotic shock is a basic biological phenomenon previously identified from bacteria to cells in lower vertebrates. The present recognition of its vital role in renal medulla is the first indication that it is more than a curiosity in mammalian cells. Possible disorders of this system have not yet been investigated, but there are a number of poorly understood diseases of the renal medulla that should be considered. Further, the

aldose reductase system, whose function we are unravelling in the renal medulla, is implicated in complications of diabetes in eyes, nerves, and kidneys.

Annual Report of the Laboratory of Pulmonary and Molecular Immunology
National Heart, Lung, and Blood Institute
October 1, 1993 to Sept 30, 1994

Based on the successful investigations of the Section on Pulmonary and Molecular Immunology, late in the year, Dr. Leonard was appointed as the Chief of a newly formed laboratory, the Laboratory of Molecular Immunology. This report summarizes only the research efforts of Dr. Leonard's Section. Research has centered on the study of select proteins critical to the process of T-cell activation. T-cells play a central role in mounting an immune response and as such are intimately related to components of each of the three principal disease area focuses of NHLBI, given the major roles that inflammation and autoimmune responses play related to the cardiovascular, pulmonary, and hematopoietic systems. This report summarizes studies related to (1) IL-2 receptor structure and function (2) Molecular regulation of IL-2 receptor α, β, and γ chain genes, and (3) The Act-2 cytokine. The IL-2 receptor has been a longstanding focus of this group, members of which were the first to discover the existence of both the IL-2Rα and IL-2Rβ chains and to clone cDNAs encoding the IL-2Rα chain. In the previous fiscal year, the group made the major discovery that mutation of the γ chain results in X-linked severe combined immunodeficiency (XSCID) in humans, a finding with major clinical and basic research implications, as detailed below. This year, the work has been extended in major ways, including the discovery that the γ chain is in fact a common γ chain (γ_c) shared by multiple cytokine receptors. Research also relates to other components of structure, function, and molecular regulation of the IL-2Rα, IL-2Rβ, and γ_c genes. In addition, the group focused some effort on a chemotactic cytokine, denoted Act-2, that it was the first to discover in 1988. Each of these areas will be discussed in turn.

IL-2 receptor-- structure and function and the discovery that IL-2Rγ mutation results in XSCID.

The human interleukin-2 receptor is being studied to understand critical components of the T cell immune response in normal and neoplastic cells. When T-cells are activated by antigen or mitogenic lectin, both IL-2 and IL-2 receptor expression are induced. IL-2 and IL-2 receptors control the magnitude and duration of the T-cell immune response based on the amount of IL-2 produced, the levels of receptors expressed, and the time course of each of these events. Whereas low levels of intermediate affinity IL-2 receptors are expressed on resting cells, following antigen stimulation, expression of both high and low affinity IL-2 receptors is potently induced. At least three chains of the IL-2 receptor are now known to exist, namely IL-2Rα, IL-2Rβ, and γ_c. Other proteins may additionally contribute to a multichain receptor complex. In the past year, major advances have been made in a number of areas.

1. The discovery of XSCID and elucidation that the γ_c chain is a component of multiple cytokine receptors. The group had discovered the previous year that mutation of γ_c results in X-linked severe combined immunodeficiency (XSCID) in humans. Since XSCID is characterized by significantly diminished or absent T cells, these data indicated that γ_c is required for thymic maturation of T lymphocytes. Interestingly, IL-2 deficient mice and human SCID patients deficient in IL-2 production have normal levels of T cells. These data suggested that XSCID and IL-2 deficiency differ in the degree of maturation of T cells, leading the group to speculate that γ_c was a component of more than one cytokine receptor

system. In fact, the group established by a combination of chemical cross-linking studies, Scatchard analyses, and functional assays that γ_c is in fact a component of the IL-4, IL-7, and IL-9 receptors. This recognition has provided a much clearer understanding of why the defect in XSCID is so severe. Consistent with the original prediction that less severe phenotypes might also be found that result from γ_c mutations, we have now found that γ_c is also the genetic defect in a moderate X-linked combined immunodeficiency (XCID).

2. IL-2, tyrosine kinases, and apoptosis. The group had previously developed a powerful system for studying IL-2 signaling, namely the transfection of 32D myeloid progenitor cells with wild type or mutant IL-2Rβ constructs. 32D cells are normally dependent on IL-3, but when transfected with IL-2Rβ, they also respond to IL-2. 32D-β cells are therefore valuable for studying IL-2 signal transduction since these cells respond to IL-2 but do not require maintenance in IL-2 for survival. Inhibitors of tyrosine kinases such as herbimycin A inhibited both IL-2 induced proliferation and IL-2 induced IL-2Rα mRNA expression in 32D-β cells, indicating that tyrosine kinase(s) are required for IL-2 induced signaling. The group found that tyrosine kinase inhibitors not only are capable of inhibiting proliferation in these cells but can also regulate apoptosis and bcl-2 expression in these growth factor dependent cells. Specifically, either IL-2 or IL-3 can upregulate bcl-2 mRNA levels; this upregulation is blocked by herbimycin A. Transfection of a bcl-2 expression vector not only prolongs survival following growth factor withdrawal but also confers resistance to herbimycin A. Thus, PTKs are involved in the regulation of apoptosis and bcl-2 expression, but herbimycin sensitive PTKs appear not to be required for the action of Bcl-2 since Bcl-2 can exert its growth suvival effect even when these PTKs are inactivated.

3. Heterodimerization of IL-2Rβ and γ_c is required for IL-2 signaling. Using the 32D system and chimeric receptor constructs, the group demonstrated that heterodimerization of IL-2Rβ and γ_c is required for IL-2 signaling. Specifically, it was shown that if cells were transfected with constructs in which the extracellular domains of IL-2Rβ and γ_c were replaced with that from IL-2Rα, then antibodies to IL-2Rα could trigger proliferation when mixtures of the α/β and α/γ_c chimeric constructs were used but not when either was expressed by itself. These and experiments with bispecific antibodies demonstrated that heterodimerization of the IL-2Rβ and γ_c cytoplasmic domains was necessary and sufficient for signaling. Other experiments indicated that the transmembrane domain of IL-2Rβ was not required and that IL-2 binds monomerically and monovalently to IL-2Rα.

4. Elucidation of IL-2 signaling pathways and their relationship to XSCID. In view of the need for heterodimerization of IL-2Rβ and γ_c for signaling, and since IL-2 rapidly induces tyrosine phosphorylation of cellular substrates, we investigated the ability of IL-2Rβ and γ_c to associate with Janus family tyrosine kinases. Indeed, we found that IL-2Rβ and γ_c associate with two different Janus family kinases (JAK1 and JAK3, respectively for IL-2Rβ and γ_c). Moreover, IL-2 induces the tyrosine phosphorylation and activation of each of these kinases. Thus, it appears that the basis for the requirement for heterodimerization might be the requirement to coordinate JAK1 and JAK3. Truncations of γ_c found in many XSCID patients are predicted to abrogate its ability to interact with JAK3, since deletion of as few as 48 amino acids is sufficient to abrogate γ_c-JAK3 interactions. Moreover, we have identified a patient with a moderate X-linked combined immunodeficiency (characterized by diminished IL-2 responses) whose γ_c gene contains a missense mutation resulting in a single amino acid change in the cytoplasmic domain. Since this mutation decreases

association with JAK3, we hypothesize that JAK3 activation is essential for intrathymic maturation and/or selection of T cells and that XSCID results from γ_c mutations that interfere with cytokine binding to γ_c and/or the ability of γ_c to associate with JAK3. If this hypothesis is correct, it is predicted that JAK3 will be found in some autosomal recessive cases of immunodeficiency that are phenotypically similar to XSCID or XCID.

5. Gene therapy for XSCID. Energies have also focused on the development of gene therapy for XSCID. To investigate the feasibility of gene therapy, an amphotropic γ_c retrovirus has been constructed as well as a γ_c adeno associated viral construct. The retrovirus is capable of successfully transducing murine fibroblasts. In addition, bone marrow and cord blood cells have been "tagged" successfully by neomycin resistance. Continued studies are underway to develop better evaluate the potential feasibility of using the viruses that have been made for gene therapy.

6. Development of a murine model for XSCID. To facilitate the preparation of a murine model of XSCID, we wish to develop a knockout mouse by homologous recombination. The murine γ_c cDNA was cloned and sequenced and used to identify genomic phage clones. The entire murine γ_c gene has now been identified, cloned, and sequenced from a genomic library prepared from 129 strain murine cells. An appropriate targeting vector was prepared, ES cells transfected and homologously recombined cells were selected in which the γ_c has been deleted. Following injection of blastocysts with the ES cells, chimeric mice have been obtained, with the hope of obtaining γ_c -/- mice in the near future. These mice should prove extremely valuable in further understanding the biology of contributions of γ_c to T cell development.

7. IL-2 receptors on monocytes and polymorphonuclear neutrophils (PMNs). We have reported that PMNs express IL-2Rβ mRNA and protein and can respond to IL-2. These cells express typical intermediate affinity IL-2 receptors, binding IL-2 with a K_d of 1.1 nM. Not only are receptors present, but IL-2 can induce augmentation of TNF-α production, with increased cytotoxicity to tumor target cells. In addition to the previous demonstration of IL-2Rβ, it has now been shown that the cells also express γ_c. Monocytes also express γ_c, and interestingly, γ_c is inducible on these cells (at both the protein and mRNA levels) in response to IL-2 and γ-interferon.

Significance to biomedical research:

These findings on IL-2 receptor structure and function contribute significantly to an understanding of the biology of this system. The finding that mutation of γ_c results in XSCID has had profound implications in T cell immunology and thymic development and led to the demonstration that γ_c is a component of multiple cytokine receptors. The studies on heterodimerization were instrumental in understanding the interaction of IL-2Rβ and γ_c with JAK1 and JAK3, respectively. Our studies have extremely important implications in basic science as well as pointing the clear direction to achieve prenatal and post-natal diagnosis, carrier female identification, and gene therapy for XSCID, and thereby have opened many new projects in basic research and clinical medicine.

Interleukin-2 receptor – molecular regulation

This laboratory was the first to analyze the promoters of each of the three chains of the IL-2 receptor, namely IL-2Rα, IL-2Rβ, and γ_c. IL-2Rα and IL-2Rβ are significantly regulated at the level of transcription. In resting T cells, the IL-2Rα chain is not expressed but is rapidly induced by mitogen stimulation. The β chain is expressed at low levels in resting T cells, but is also readily inducible following T cell activation. In T cells, γ_c is constitutively expressed and appears not to be very inducible, although it is more inducible in monocytes. In the past year, the group has made major advances: (1) A new enhancer in the IL-2Rα 5' regulatory region has been delineated and functional coordination between this enhancer and the previously . (2) The critical cis-acting elements in the IL-2Rβ promoter were delineated and three regions with enhancer activity have been characterized. (3) Major progress has been made in identifying transcription factors responsible for the regulation of expression of both the IL-2Rα and IL-2Rβ chain genes. A new PMA inducible transcription factor has been characterized and rapidly induced latent transcription factors (known as STAT proteins for signal transducers and activators of transcription) have been found to induced by a number of cytokines. These findings therefore are critical not only to an understanding of IL-2 receptor gene regulation but also extend to the regulation of other T-cell activation genes.

IL-2Rα chain gene regulation: The IL-2Rα chain 5' regulatory sequences were previously delineated by this group, with characterization of an enhancer element which spans an NF-κB site, a CArG motif, an Sp1 site, and a fourth site denoted NF-IL-2RA, all located between -299 and -228 relative to the major transcription initiation sites. Given how far this region was from the promoter and its relatively poor conservation in mouse, the possibility that other important elements would exist further downstream was investigated, leading to the discovery of a second essential positive element. This element contains binding sites for at least two transcription factors-- Elf-1 (an Ets family protein) and HMG-I/Y (11-12 kDa) high mobility group proteins that bind in the minor groove of DNA and are highly expressed in rapidly proliferating cells. Elf-1 is critical for IL-2Rα promoter activity. A single nucleotide change within the Elf-1 site is sufficient to abrogate IL-2Rα promoter activity. Although HMG-I(Y) and Elf-1 can physically associate with each other in vitro, HMG-I(Y) does not appear to play a role in augmenting Elf-1 binding. Instead, it likely plays a role in bending DNA, perhaps promoting protein-protein interactions. A binding site selection analysis is being performed to elucidate optimal binding sites for this factor. Enhancer and promoter constructs are being evaluated to further elucidate the role played by these proteins. Identification of the new enhancer provides new insights into the protein-protein and protein-DNA interactions that regulate cell type specific and inducible IL-2Rα gene activitation.

In parallel, experiments using *in vivo* footprinting methodology have been pursued. These experiments were motivated by the observation that there appear to be at least two different molecular mechanisms for activating the IL-2Rα gene. Specifically, mutation of the κB site results in a loss of IL-2Rα-CAT activity in HTLV-I infected cells but not in Jurkat cells, whereas mutation of the CArG motif (which can bind serum response factor *in vitro*) appears to be important in both cell types but moreso in PMA stimulated Jurkat cells. Since there is no PMA-induced binding to the CArG motif *in vitro*, in Jurkat, it is striking that PMA induced binding to the CArG motif was found *in vivo*.

IL-2Rβ chain gene regulation: The 5'-flanking region of the human IL-2Rβ gene was previously reported by this group to possess promoter activity. In the past year, the group

reported nested 5' deletion and internal deletion mutants as a means of delineating cis-acting elements critical for basal and PMA-inducible IL-2Rβ promoter activity. The region downstream of -363 is critical for both basal and PMA inducible expression and contains at least three enhancer-like regions. The -56 to -34 enhancer was the most potent and had high level activity in two T cell lines but not in HeLaS3 cells. This enhancer contains a GGAA Ets binding site. Ets family members encode transcription factors that interact in sequence-specific manner with purine-rich motifs in promoters and enhancers of viral and cellular genes encoding important immunological regulatory proteins, including the polyomavirus enhancer, the moloney sarcoma virus (MSV) long terminal repeat (LTR), the human T-cell receptor (TCR) α-chain enhancer, the IL-2 enhancer and the class II MHC promoter. The IL-2Rß Ets site binds Ets-1 and GABP in vitro, two different Ets family proteins. This conclusion is based on experiments using electrophoretic mobility shift assays, DNaseI footprinting, and methylation interference analyses. Mutation of the Ets motif strongly diminished both promoter and enhancer activities. Thus, this site plays a key role in regulating basal and PMA-inducible IL-2Rβ activitiy and may also contribute to tissue specific expression of the IL-2Rβ gene. The group has also begun to identify proteins binding to the other enhancers. In one of these, a PMA inducible binding activity has been found which appears distinct from AP-1 and NF-κB. This PMA inducible factor has been characterized. Since IL-2Rβ mRNA is potently induced in normal human peripheral blood lymphocytes by the protein kinase C activator PMA, the T cell mitogen PHA and anti-CD3 antibodies, and somewhat less strongly induced by IL-2, it is reasonable to believe that the inducible enhancers that have been identified play a role in these physiological modes of induction of the IL-2Rβ gene.

STAT proteins. In view of the findings of the role of Janus family kinases in IL-2 signaling, the group has become particularly interested in investigating the activation of STAT proteins by cytokines that use γ_c. These latent transcription factors are rapidly induced by tyrosine phosphorylation. The lab has in fact evaluated the abilities of IL-2, IL-4, IL-7 (which use γ_c) as well as IL-13 and have found that all induce STAT proteins. This is an area of active investigation.

Significance of study to biomedical research: These findings on IL-2Rα, IL-2Rβ, and γ_c contribute significantly towards an elucidation of the process of T-cell activation given the central role played by these genes in regulating the T-cell immune response. The identification of specific transcription factors and signals that regulate these genes contributes significantly to our understanding of and our ability to manipulate the T-cell

immune response.

Studies related to the Act-2 cytokine:

In 1988, the group discovered Act-2 as a gene which was expressed in activated but not resting T cells. It was found to encode a secreted protein of 69 amino acids in length. Act-2 is a member of a family of small secreted proteins, many members of which have inflammatory or chemotactic activities. This family, whose members are now denoted as "chemokines" can be divided into two subfamilies based on whether the first two of four conserved cysteines are adjacent (CC) or separated by one amino acid (CXC). Act-2 is a member of the CC subfamily, and is presumed to be the human homologue of murine macrophage inflammatory protein (MIP)-1β. Synthesis and secretion of this cytokine are rapidly induced in T cells, B cells, and monocytes following stimulation with antigen or mitogen. Act-2 has recently been demonstrated by others to be specifically chemotactic for CD4+ T lymphocytes and by our group to have activity as a bone marrow stem cell inhibitor and can efficiently compete with murine MIP-1α for binding. During the past year, we determined the three dimensional structure of Act-2/hMIP-1β by multimensional NMR. To accomplish this project, it was necessary to produce in large quantities unlabeled, ^{15}N-labeled, and ^{15}N plus ^{13}C doubly labeled Act-2/hMIP-1β protein.

Act-2/hMIP-1β is a symmetric homodimer with a relative molecular mass of 16 kDa. The structure of the monomer is similar to that of the related CXC chemokine interleukin-8. However, the quaternary structures of the two proteins are entirely distinct, and the dimer interface is formed by a completely different set of residues. Whereas the IL-8 dimer is globular, the Act-2/hMIP-1β dimer is elongated and cylindrical. These data are extremely interesting in that they provide a rational explanation for the absence of cross-reactivity between the two chemokine subfamilies in terms of receptor binding. Formation and stabilization of the two different types of dimers likely arises from the burial of hydrophobic residues, based on calculation of the solvation free energies of dimerization. Since Act-2 is an abundantly and rapidly synthesized protein following T-cell activation with at least two known biological activities (chemotactic for $CD4^+$ T cells and a stem cell inhibitor), this is an important area of investigation. Solving the structure of Act-2/hMIP-1β represents the first solution of a structure for a CC chemokine. Comparison to the structure of members of the CXC part of the family of small secreted proteins explains why these two groups of chemokines utilize different receptors and paves the way towards the development of agonistic and antagonistic pharamaceuticals.

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